THE MOLECULAR BIOLOGY OF THE PHOSPHATE-SPECIFIC TRANSPORT SYSTEM OF *ESCHERICHIA COLI* K12

by

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STATEMENT

The work described in this thesis is the candidate's own, except where specifically stated otherwise in the text.

Brian Surin

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PREFACE

The work presented in this thesis was performed between October 1982 and September 1985 in the Department of Biochemistry, John Curtin School of Medical Research, Australian National University, Canberra, under the supervision of Dr. Harry Rosenberg and Dr. Graeme B. Cox. Financial support between January 1982 and November 1985 was by an Australian National University Postgraduate Research Award. Parts of this work have been published.

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This thesis is divided into eight chapters. The General Introduction (Chapter 1) contains references to Literature up to September 1985. Other relevant publications which appeared after this date have been included in later Chapters. Where unpublished information from other workers has been included, it is acknowledged as a personal communication. The materials and methods are described in Chapter 2. This is followed by five

chapters describing the results of experiments, each chapter containing a brief introduction and discussion. The final chapter embodies a general discussion of the results and speculation on their implications.

A problem was encountered in the use of a single mnemonic to describe the genes comprising the Pst system, in that two of the genes had the mnemonic pho (phoS and phoU), while the other genes had the designation pst (pstCAB). For the sake of brevity the use of the mnemonic pst was adopted (as in "pst gene", "pst region"), unless otherwise stated, to identify a genetic component (s) of the Pst system in a general sense. Consequently the designation pstS was used instead of phoS in Chapter 8.

The following conventions were used when a strain and/or plasmid were mentioned in the text. For a strain, the genotype of the strain considered relevant to the particular experimental manipulation was presented. For a plasmid-bearing strain, it was decided to include the strain number and the plasmid carried, followed by the relevant chromosomal allele, viz., strain HR300 (pAN356/Δ[tna-glmS]). Only where specific and complex manipulations of a plasmid are described, is the relevant plasmid genotype presented, viz., pAN356 ($phoS^+pstC^+A^+B^+phoU^+$). The strains and plasmids of Tables 2.1 and 2.2 are listed in increasing alphabetical order, and in increasing numerical order in a particular alphabetical group. The thesis conforms as far as possible to the recommendations of the Biochemical Journal.

LIST OF ABBREVIATIONS USED

A_{595}, A_{660}	absorbance at the indicated wavelength in nm
ADP	adenosine 5'-diphosphate
ATP	adenosine 5'-triphosphate
ATPase, F_1F_0-ATPase	adenosine triphosphatase
bisacrylamide	N,N'-methylene bisacrylamide
bp	base pair
cAMP	adenosine 3',5'-cyclic monophosphate
CCCP	carbonylcyanide m-chlorophenylhydrazone
CIP	calf intestinal alkaline phosphatase
CTP	cytidine 5'-triphosphate
$\Delta\tilde{\mu}_{H^+}$	electrochemical potential of hydrogen ions across the cell membrane
ΔpH	pH difference across the inner membrane
$\Delta\psi$	electrical potential difference across the cell membrane
Δp, pmf	protonmotive force
2,3-DHB	2,3-dihydroxybenzoic acid
DNA	deoxyribonucleic acid
DNP	2,4-dinitrophenol
DTT	D,L-1,4-dithiothreitol
EACA	ε-amino-n-hexanoic acid (or ε-amino-n-caproic acid)
EDTA	ethylenediaminetetraacetate
EGTA	ethyleneglycoltetraacetate
F_1-ATPase	soluble ATPase capable of being removed from the membrane
F_0	the membrane-sector of the ATPase

GTP	guanosine 5'-triphosphate
kb	kilobase pair
kDa	kilodalton
Km^{app}	the apparent Michaelis constant
LBT	Luria broth containing thymine
MBP	maltose-binding protein
M_r	relative molecular mass
ninhydrin	indane-1,2,3-trione hydrate
NP-40	Nonidet P-40; octylphenoxy-polyethoxy-ethanol, containing 7-9 mol/mol of ethoxy linkages
ORF	open reading frame
PAB	ρ-aminobenzamidine
PBP	phosphate-binding protein
PEP	phosphoenol pyruvate
Pi	inorganic phosphate
Pit	phosphate (inorganic) transport
Pst	phosphate-specific transport
PTS	phosphoenolpyruvate:sugar phosphotransferase system
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
STEM	buffer containing sucrose, Tes, EGTA and magnesium acetate (Chapter 2, Section 2.3C)
TBE	buffer containing Tris, boric acid and EDTA (Chapter 2, Section 2.5A)
TE	buffer containing Tris and EDTA (Chapter 2, Section 2.6A)

TEA	buffer containing Tris-acetate, sodium acetate and EDTA (Chapter 2, Section 2.5B)
Temed	N,N,N',N'-tetramethylethylenediamine
Tes	2-{(2-hydroxy-1,1-bis(hydroxymethyl)ethyl) amino}-ethanesulphonic acid
Tris	tris(hydroxymethyl)aminoethane
TTP	thymidine 5'-triphosphate
UTP	uridine 5'-triphosphate
UV	ultraviolet
wt	weight

ABSTRACT

The phosphate-specific transport (Pst) system of Escherichia coli K12 was studied with a combination of genetic and biochemical techniques. A DNA fragment which complemented all mutations mapping in the pst region of the chromosome was sequenced. Open reading frames within the DNA sequence were identified and related to known mutant alleles by genetic complementation. The proteins encoded by the open reading frames were also identified. Manipulation of chromosomal DNA cloned into plasmids enabled the characterization of the role of particular genes with respect to the known functions of the Pst system: inorganic phosphate (Pi) transport and regulation of the pho regulon.

Five open reading frames, comprising the pstS (previously phoS), pstC, pstA (= phoT), pstB and phoU genes, were identified in the pst region. The pstC gene had not been described hitherto. The first four genes are essential for Pi transport and are also involved in the regulation of the pho regulon, while the phoU gene appears to be only regulatory. The proteins encoded by the pstC, pstB and phoU genes were found to be peripherally located on the inner membrane. The PstA protein was deduced to be an integral inner membrane protein on the basis of its hydrophobicity and its predicted secondary and tertiary structure. A periplasmic location had previously been reported for the phosphate-binding protein, the pstS gene product. The PhoU protein was purified from a strain which produced this protein in high quantity. Amino acid analysis of the purified

protein confirmed the reading frame proposed for the phoU gene.

The similarities between the Pst system and other shock-sensitive transport systems of gram-negative bacteria are discussed. The PstB protein was found to contain the consensus adenine nucleotide-binding fold. It is proposed that the PstB protein energizes substrate transport in the Pst system by binding and hydrolysis of ATP. The hydrophobic proteins, PstC and PstA, are postulated to be directly involved in the transport of Pi across the inner membrane.

The role of the Pst system in the regulation of the pho regulon is discussed. It is proposed that the Pst system regulates gene expression in the pho regulon through the mediation of the PhoU protein.

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CHAPTER 1

GENERAL INTRODUCTION

1.1 THE STRUCTURE OF THE GRAM-NEGATIVE CELL ENVELOPE

The cytoplasm of the gram-negative bacterial cell is surrounded by a complex cell envelope (see Beveridge, 1981) which consists of three layers (see Fig. 1.1). The cytoplasmic (or inner) membrane consists of a bilayer of lipid in which proteins are embedded. It separates the cytoplasm from the cell wall, which is composed of a layer of peptidoglycan enclosed by the outer membrane. The peptidoglycan consists of a network of polysaccharides covalently cross-linked by short peptides. The outer membrane contains phospholipid, lipopolysaccharide and proteins arranged in a bimolecular leaflet (see Lugtenberg and van Alphen, 1983) and is covalently attached to the peptidoglycan layer through lipoprotein. The periplasmic space, contained between the inner and the outer membrane, has until recently been considered an aqueous compartment. However Hobot *et al.* (1984) have suggested that the peptidoglycan fills the entire periplasmic space, forming a gel.

The three layers of the cell envelope have distinct functions. The peptidoglycan forms a rigid layer which enables the cell envelope to withstand the osmotic pressure exerted by the cytoplasm, and determines the shape of the cell. The periplasmic space contains soluble proteins which are involved in the degradation of solutes and turnover of structural components

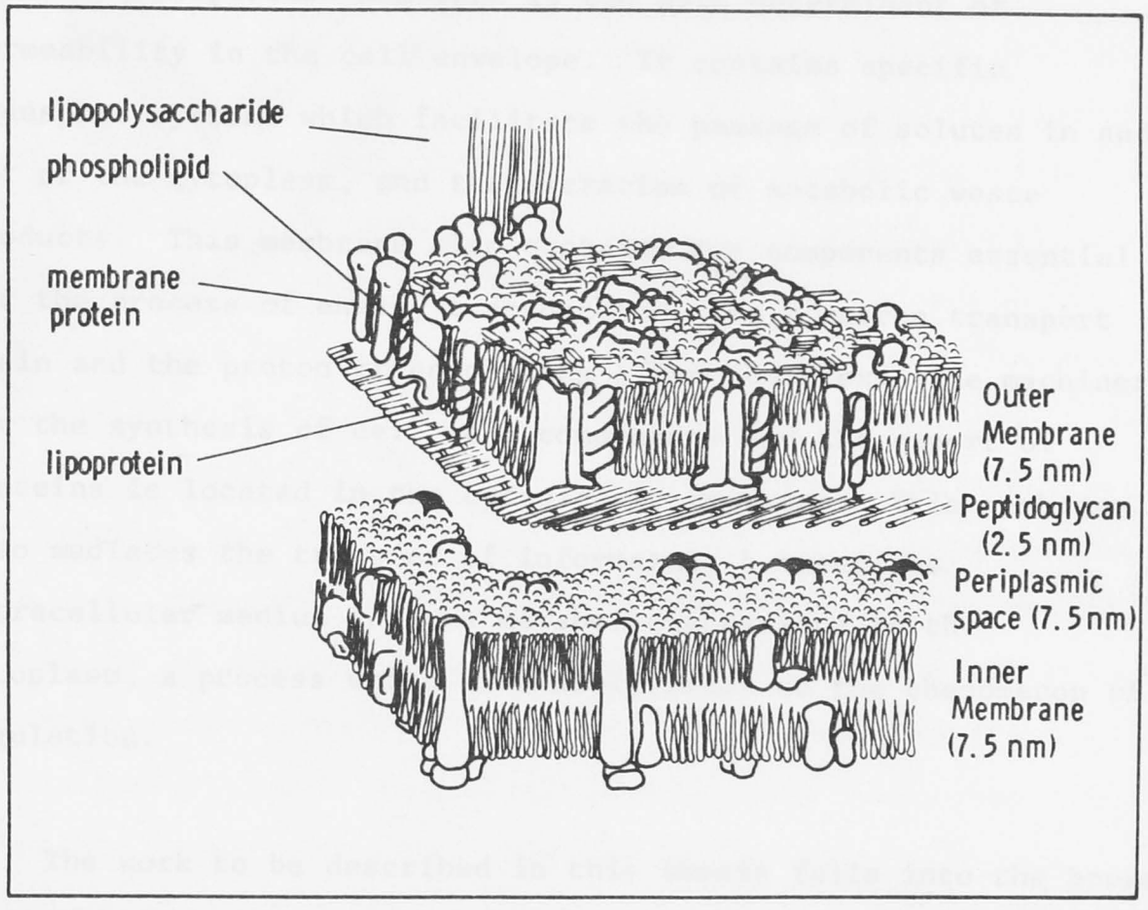


Fig. 1.1. Structure of the gram-negative cell envelope. Reproduced with the kind permission of Dr. Robert Glass, from: Glass, R.E. (1982) Gene Function (Croom and Helm, London).

of the cell envelope, in the perception of chemotactic stimuli, and in the transport of solutes across the cell envelope.

The cytoplasmic membrane is the main determinant of permeability in the cell envelope. It contains specific transport systems which facilitate the passage of solutes in and out of the cytoplasm, and the excretion of metabolic waste products. This membrane also contains two components essential for the process of energy transduction, the electron transport chain and the proton-translocating ATPase complex. The machinery for the synthesis of cell wall components and the export of proteins is located in the cytoplasmic membrane. This membrane also mediates the transfer of information between the extracellular medium and the metabolic apparatus in the cytoplasm, a process which ultimately leads to the phenomenon of regulation.

The work to be described in this thesis falls into the broad area of solute transport across the cell envelope. Since the advent of rapid filtration techniques two factors have been largely responsible for the rapid progress in this field of research in the last 25 years. The first factor was the development of methods for producing membrane vesicles (Kaback, 1971). The use of membrane vesicles circumvented many of the difficulties of working with intact cells, such as endogenous energy reserves and metabolism of the substrate after translocation, while retaining the essential feature of a transport process: the transfer of a substrate from one compartment to another across a membrane. The second factor was

the proposal by Mitchell (1961) of the chemiosmotic hypothesis.

1.2 THE CHEMIOSMOTIC HYPOTHESIS

Studies on the transport of phosphate in bacteria led Mitchell to consider mechanisms by which phosphate (Pi) could pass through a biological membrane and be released in a free form inside the cell. A postulate introduced by Mitchell (1963) that was to prove important in the formulation of the chemiosmotic hypothesis was the concept of vectorial metabolism. If an enzyme is embedded within a membrane separating two compartments, substrate can interact with the enzyme from one compartment only, and products can leave into the other (see the description of the phosphoenolpyruvate:sugar phosphotransferase system in Section 1.3B). It is a simple conceptual step from transmembrane enzymes to transmembrane transport proteins, which have no apparent catalytic function in a chemical reaction, but which bind substrate on one side of the membrane and release it on the other.

The two ideas that led to the proposal of the chemiosmotic hypothesis were: (1) that electron transfer in the respiratory chain or in the photosystems of photosynthetic organisms leads to a separation of hydrogen and hydroxyl ions on opposite sides of the inner membrane, and (2) that the ATPase associated with the inner membrane is anisotropic and that it utilizes this gradient of hydrogen (or hydroxyl) ions to synthesize ATP from ADP and Pi (Mitchell, 1961). In other words, respiration (or photosynthesis) and the synthesis of ATP can be chemiosmotically

coupled, via the gradient of hydrogen ions across the membrane, in the process of oxidative phosphorylation. This gradient gives rise to the electrochemical potential of hydrogen ions across the membrane, $\Delta\tilde{\mu}_H+$, which has two components. These are $\Delta\psi$, the electrical potential difference across the membrane caused by the separation of charge, and ΔpH, the pH gradient across the membrane. $\Delta\tilde{\mu}_H+$, $\Delta\psi$ and ΔpH are related in the following equation (Rosen and Kashket, 1978):

$$\Delta\tilde{\mu}_H+ = F\Delta\psi - 2.3RT\Delta pH$$

where F is the Faraday constant, R is the gas constant, and T is the temperature in degrees Kelvin. By analogy with the electromotive force, emf, of electrochemical cells, Mitchell refers to the electrochemical potential difference of protons across the membrane as the protonmotive force, pmf, or Δp, as follows (Mitchell, 1966):

$$\Delta p = \Delta\tilde{\mu}_H+/F = \Delta\psi - 2.3RT\Delta pH/F$$

$$= \Delta\psi - Z\Delta pH$$

where Z is a combination of constants equal to about 59 mV/pH unit at 37°C. In the steady-state the gradient of hydrogen ions is oriented such that $\Delta\psi$ is positive outside, and ΔpH is alkaline inside the bacterial cell.

1.2A THE CHEMIOSMOTIC HYPOTHESIS AND ACTIVE TRANSPORT

It is now generally accepted that the chemiosmotic hypothesis provides a logical basis for the description of the coupling of energy to active transport (Harold, 1977a; Rosen and Kashket, 1978). Two classes of chemiosmotically coupled transport systems are recognized, primary and secondary (Mitchell, 1967; Harold, 1977b). Primary translocations are those in which movement is linked to either chemical or photochemical energy. Examples of primary transport systems are the respiratory chains, photosynthetic systems, and bacteriorhodopsin (Harold, 1977a). The proton-translocating ATPase functions as a primary transport system only when it couples proton extrusion with the hydrolysis of ATP. When the ATPase catalyses the phosphorylation of ADP, coupled to the influx of protons into the cell, it is performing a secondary translocation (Rosen and Kashket, 1978).

Secondary translocations are those in which movements are linked to an electrical potential, a concentration gradient, or both. The catalysts for these movements are known as porters, to distinguish them from the enzymes of the primary transport systems. Mitchell (1967) proposed three different types of secondary transport systems:

(i) uniports, which catalyse the transport of substrate across the cell membrane in response to a concentration gradient of the substrate if it is uncharged (facilitated diffusion), or to the membrane potential if it is charged;

(ii) symports, where the transport of a particular substrate is obligatorily linked to the movement of another substrate in the same direction on the same porter, the second substrate moving down its potential gradient; and

(iii) antiports, where the transport of substrate is coupled to the movement, along the same porter but in the opposite direction, of a second substrate down its potential gradient. These secondary transport systems can make use of the proton gradient that is produced by the primary systems of the respiratory chain and photosynthesis.

1.3 TYPES OF BACTERIAL TRANSPORT SYSTEMS

Bacteria have evolved several different mechanisms for the transport of solutes across the cell envelope. Four such systems are known to mediate solute transport through the use of a carrier protein (s). These are the facilitated diffusion, group translocation, osmotic shock-resistant and osmotic shock-sensitive systems. Since this thesis is concerned mainly with a shock-sensitive transport system, I intend to discuss very briefly the other types of transport system found in bacteria. There are several reviews which comprehensively cover the different bacterial transport systems (Dills *et al.*, 1980; Hengge and Boos, 1983; Kaback, 1983).

1.3A FACILITATED DIFFUSION

In facilitated diffusion, the simplest form of transport

across a bacterial cell membrane, a substrate moves down its concentration gradient in a process mediated by a carrier protein located in the membrane. This type of system is not coupled to metabolic energy and under normal circumstances it would maintain an equilibrium of its substrate across the membrane. This situation does not arise, however, because enzymatic modification of the transported substrate effectively "traps" it inside the cell. In E. coli the uptake of glycerol occurs by facilitated diffusion (Richey and Lin, 1972). The glycerol facilitator protein mediates the passage of glycerol across the cell membrane. After entering the cell glycerol is phosphorylated by an enzyme located in the cytoplasm, glycerol kinase (Hayashi and Lin, 1965).

1.3B GROUP TRANSLOCATION

The phosphoenolpyruvate:sugar phosphotransferase system (PTS) (Dills et al., 1980) is probably the best known system of this type. This system is composed of some soluble cytoplasmic enzymes and several membrane-associated proteins which catalyze the transport and concomitant phosphorylation of various hexoses and hexitols. The sequence of reactions involved in PTS-mediated uptake, as found in E. coli, is shown below (From a review by Rosen and Kashket, 1978):

(1) PEP + HPr $\xrightleftharpoons{\text{enzyme I}}$ phospho-HPr + pyruvate

(2) Phospho-HPr + sugar $\xrightarrow[\text{complex}]{\text{enzyme II}}$ sugar phosphate + HPr

(1) + (2) PEP + sugar $\xrightarrow{\text{PTS}}$ sugar phosphate + HPr

Enzyme 1 catalyses the phosphoryl transfer from phosphoenolpyruvate (PEP) to a histidinyl residue of the "histidine protein", HPr. Enzyme I and HPr are common to all PT-systems. The enzyme II complex, which catalyses the phosphoryl transfer from phospho-HPr to a glycosyl unit, serves as the sugar recognition component of the PT-system: there is a family of enzyme II proteins. The phosphorylation of the sugar is obligatorily linked to transport. This is in direct contrast to the uptake of glycerol by facilitated diffusion (Section 1.3A) where phosphorylation occurs after the solute has been taken up into the cell.

In addition to its role in the transport of PT-sugars, the PTS regulates the utilization of sugars which are transported by other systems. The PTS regulates the uptake of solutes, which are required to induce the synthesis of specific catabolic enzymes (inducer exclusion), and it regulates the synthesis of these catabolic enzymes by controlling the activity of the enzyme adenylate cyclase and thus the level of cAMP (PT-mediated catabolite repression). This cyclic nucleotide is involved in the initiation of transcription of mRNA from certain catabolite-sensitive operons (Botsford, 1981). A central role in this regulation is attributed to factor III^{glc}, a peripheral membrane protein which together with the integral membrane protein II^{glc} effects the transport and phosphorylation of glucose and methyl α-glucoside. Factor III^{glc} can exist in two forms: phosphorylated and non-phosphorylated. Phosphorylation of III^{glc} is catalysed by enzyme I and HPr. In its non-phosphorylated form, III^{glc} is an inhibitor of a number of non-PTS uptake systems. This form of

III^{glc} has been shown to bind to and inactivate the lactose permease (Osumi and Saier, 1982; Nelson et al., 1983), and glycerol kinase (Postma et al., 1984). Phosphorylated III^{glc}, on the other hand, is thought to activate adenylate cyclase. Consequently the intracellular level of cAMP will increase, and this will stimulate the expression of the genes encoding transport systems for the uptake of non-PT substrates (e.g. lactose, glycerol, maltose and melibiose).

1.3C ACTIVE TRANSPORT SYSTEMS

The first indication of the diverse nature of active transport systems in gram-negative bacteria was provided when Dreyfuss and Pardee (1965) showed that sulphate transport in S. typhimurium was almost abolished by conversion of whole cells to spheroplasts using a sequential lysozyme/EDTA treatment, an observation contrasting that of Sistrom (1958) who reported that this treatment did not interfere with β-galactoside accumulation.

The development of the osmotic shock procedure (Neu and Heppel, 1965) was an important factor in the resolution of the types of bacterial transport systems. In this procedure cells suspended in a concentrated sucrose solution at room temperature were rapidly dispersed in about 80 volumes of a cold dilute solution of $MgCl_2$. This resulted in the release of a group of proteins located near the cell surface, including alkaline phosphatase.

It was subsequently demonstrated (Pardee et al., 1966) that

sulphate transport in S. typhimurium was also severely inhibited by osmotic shock. The inhibition corresponded to the loss fron the cells of a sulphate-binding activity which could be recovered in the shock fluid. This observation has since been demonstrated for other transport systems (see Rosen and Heppel, 1973), and these systems have been designated shock-sensitive. In contrast there are several permeases which are relatively insensitive to osmotic shock (Heppel et al., 1972). Both types of transport system are considered to reflect "true" active transport, in the sense that substrate is not altered during the translocation step.

1.3C1 SHOCK-RESISTANT ACTIVE TRANSPORT SYSTEMS

Shock-resistant active transport systems share several characteristic features (Berger and Heppel, 1974) such as high sensitivity to protonophores (e.g. DNP, CCCP) which collapse $\Delta\tilde{\mu}_H+$, and mild sensitivity to arsenate, an inhibitor which is known to lower the intracellular level of ATP. Shock-resistant systems can use ATP generated by the process of substrate level phosphorylation during glycolysis, in the presence of a functional ATPase. Such systems are active in spheroplasts and membrane vesicles.

The best known transport system of this type is the lactose permease of E. coli (see Kaback, 1983). A single protein, the M protein, located in the cytoplasmic membrane, catalyses the accumulation of lactose and other β-galactosides. Since the original proposal of Mitchell (1963) that lactose transport

occurs in symport with protons, ample evidence has been accumulated to support the proposal. West (1970) and West and Mitchell (1972, 1973) demonstrated that addition of lactose to de-energized cells caused the alkalinization of the external medium. Subsequent experiments with intact cells (Flagg and Wilson, 1977), membrane vesicles (Schuldiner and Kaback, 1975), and proteoliposomes reconstituted with purified M protein (Newman et al., 1981; Foster et al., 1982), have provided virtually unequivocal evidence that $\Delta\tilde{\mu}_{H}+$ or one of its components is the immediate driving force in the transport of lactose and β-galactosides.

The rate of uptake of lactose, observed in reconstituted proteoliposomes, is lower than that of membrane vesicles. Together with the results of genetic studies this would indicate that an additional protein (s), common to all shock-resistant systems, is involved in lactose transport (see Hengge and Boos, 1983). Carrasco et al. (1984) reported that the topology of the C-terminus of the M protein reconstituted in proteoliposomes is different from that seen in membrane vesicles, an observation that could explain the lower rate of uptake in the reconstituted system. More work is needed to resolve this controversy.

Many shock-resistant systems which transport their substrates by a proton-symport mechanism are now recognised. These include transport systems for amino acids, carboxylic acids, sugars, organophosphate esters and inorganic ions (see Harold, 1977a).

Active transport of solutes can be coupled to gradients of cations other than protons. A sodium-melibiose symport in E. coli (Tsuchiya et al., 1977) has been reported. It was shown that proton efflux through the primary transport systems provided a driving force for the uptake of sodium ion and thiomethyl-β-D-galactopyranoside, a non-metabolizable sugar analog transported by this system.

It has been stated by Drachev et al. (1985) that $\Delta\tilde{\mu}_{H}+$ can be buffered by Na^+ and K^+ gradients, which can be used either directly (e.g. in melibiose transport) or indirectly by conversion to $\Delta\tilde{\mu}_{H}+$.

1.3C2 SHOCK-SENSITIVE ACTIVE TRANSPORT SYSTEMS

Shock-sensitive active transport systems are severely affected by a cold osmotic shock (Neu and Heppel, 1965), mainly due to the loss of substrate-binding proteins which are located in the periplasmic space and are essential to these systems (see Heppel, 1969). Shock-sensitive systems are characterised (Berger and Heppel, 1974) by a fairly severe inhibition of transport activity by arsenate, while displaying a mild sensitivity to protonophores. These systems do not require the presence of a functional ATPase complex when supplied with a source of "glycolytic" ATP. Furthermore ATPase defective mutants cannot use energy derived from the oxidative reactions of the electron transport chain to drive shock-sensitive transport. These transport systems do not operate in spheroplasts and membrane vesicles. Although shock-sensitive transport systems have been

extensively researched, the mode of energization of these systems has still not been firmly established.

As shock-sensitive systems only function in intact cells, it is necessary to eliminate the endogenous energy reserves which would complicate any attempt at studying the mode of energization by the addition of potential energy sources to the extracellular medium. Koch (1971) achieved this by allowing cells to transport α-methylglucoside in the presence of azide, which stimulates this transport, until the reserves are eliminated. Berger (1973) incubated cells with 2,4-dinitrophenol, an uncoupler of oxidative phosphorylation. In an attempt to maintain the energized state of the cytoplasmic membrane the cells depleted their endogenous reserves of energy. After this treatment the cells were totally dependent on externally added energy sources, and the ability of these energy sources to drive transport could be examined. Berger (1973) and Berger and Heppel (1974) compared the ability of D-lactate and glucose to stimulate transport in energy-depleted normal cells and ATPase defective mutants, in the presence and absence of uncouplers and metabolic inhibitors. The authors showed that shock-sensitive glutamine transport was energized by phosphate-bond energy, while shock-resistant proline transport required the protonmotive force. A similar result was obtained in a comparison between proline transport and the shock-sensitive transport of isoleucine (Kobayashi et al., 1974), and by Hong et al. (1979), who studied the transport of glutamine and proline.

The nature of the donor of phosphate-bond energy proposed to

drive these systems has not been established. The use of strains deficient in the synthesis of acetylphosphate, in combination with various metabolic inhibitors (Hong et al., 1979), implicated acetylphosphate as the source of phosphate-bond energy. Hunt and Hong (1981) subsequently reconstituted shock-sensitive glutamine transport by the addition of purified glutamine-binding protein to isolated membrane vesicles. In this system glutamine uptake required pyruvate/NAD or succinate as the source of energy and was inhibited by arsenate but neither ATP nor acetylphosphate were able to energise the uptake (Hunt and Hong, 1983a).

Other work on these systems indicates that phosphate-bond energy may be necessary, but not sufficient, for the uptake of solutes by shock-sensitive systems (Plate et al., 1974; Singh and Bragg, 1976, 1977; Plate, 1979). Treatments which reduce the membrane potential and, either have no effect on ATP levels (e.g. valinomycin-plus-potassium added to ATPase defective mutants) (Plate, 1979) or, lead to an increase in the level of ATP (e.g. colicin K added to ATPase defective mutants) (Plate et al., 1974), inhibit the uptake of glutamine. Singh and Bragg (1976, 1977) showed that in cytochrome-deficient mutants, which do not have a functional electron transport system, glutamine transport was dependent upon the activity of the proton-translocating ATPase. In the absence of electron transport the protonmotive force is maintained by the ATPase through the hydrolysis of ATP coupled to the pumping of protons out of the cell. Inactivation of the ATPase under these conditions would result in the abolition of the membrane potential.

Investigations into the coupling of energy for the shock-sensitive transport of galactose (Henderson et al., 1977) and arabinose (Daruwalla et al., 1981) excluded the involvement of a proton-symport mechanism in the transport of these sugars. As pointed out by the authors, however, transport of substrate in these systems could be coupled to either the membrane potential or to the movement of ions other than protons.

At the moment there is no definitive solution to the mode of energy coupling in shock-sensitive transport. The weight of evidence would seem to suggest a role for both phosphate-bond energy and the protonmotive force in the energization of substrate transport by shock-sensitive systems. The failure to observe proton-symport with substrate in these systems (Henderson et al., 1977; Daruwalla et al., 1981) would indicate that the protonmotive force is not the immediate energy source for the transport of substrate. It has been suggested (Silhavy et al., 1978) that the Δp is necessary to maintain the components of shock-sensitive systems in the cytoplasmic membrane in a functional state, while phosphate-bond energy is the driving force for the actual translocation of substrate. A similar model has been described for the transport of potassium in Streptococcus faecalis which requires both ATP and the protonmotive force (Bakker and Harold, 1980).

1.4 THE PHOSPHATE TRANSPORT SYSTEMS OF ESCHERICHIA COLI

1.4A THE PIT AND PST SYSTEMS: DISCOVERY AND DESCRIPTION

The work which eventually led to the elucidation of the

phosphate transport systems of Escherichia coli was concerned with the regulation of the synthesis of the periplasmic enzyme, alkaline phosphatase. This synthesis is phosphate-repressed, but is induced when the Pi concentration in the medium is lowered to about $5x10^{-5}$ M. Echols et al. (1961) reported the isolation of two-types of "constitutive" mutants which produced high amounts of alkaline phosphatase in media containing high concentrations of Pi. The mutations, named R1 and R2, mapped in different regions of the chromosome. The R2-type mutation, mapping at min 83 on the E. coli genetic map (Bachmann, 1983) fell into two categories, R2a and R2b (Garen and Otsuji, 1964). Strains carrying the R2a allele lacked a periplasmic protein of molecular weight about 40,000 daltons, which was present in wild strains as well as in R2b mutants. The relationship between the R2a protein and phosphate transport remained obscure, however, until Gerdes and Rosenberg (1974) purified the phosphate-binding protein. Amino acid analysis of the purified protein, and the use of immunological techniques, showed that the phosphate-binding protein (PBP) and the R2a protein were identical. The unambiguous demonstration of the restoration of Pi transport in spheroplasts by the PBP (Gerdes et al., 1977) linked the product of the R2a gene with Pi transport.

Pi transport in E. coli was first described in some detail by Medveczky and Rosenberg (1970, 1971), who showed this to be an active process. The initial rate of uptake of Pi was increased about 20-fold if the cells were "starved" of Pi by incubation in a Pi-free medium for two-hours. ^{32}Pi transported during the first minute was present in the cell largely as Pi, and could be

chased out by a ten-fold excess of non-radioactive Pi, or by arsenate. The induction, by Pi starvation, of the rapid initial transport was reminiscent of the induction of alkaline phosphatase, but the connection between these phenomena did not become clear until later.

These early studies of Pi transport were beset by conflicting and often irreconciliable results. Much of this confusion was cleared when it was shown that Pi transport in E. coli K12 was carried out primarily by two distinct uptake systems (Willsky et al., 1973). The presence of more than one system for Pi transport in E. coli was suggested by previous investigations (Bennett and Malamy, 1970; Medveczky and Rosenberg, 1971). Willsky et al. (1973) designated the two systems "Pit" (phosphate inorganic transport) and "Pst" (phosphate-specific transport). The authors also described the mutations in the R2 region in greater detail. These mutations had by then undergone a change in name: Aono and Otsuji (1968) used the mnemonic phoS for R2a and Willsky et al. (1973) suggested phoT as an alternative for R2b. They reported that transductional analysis placed phoS and phoT at 83 min, close to another mutation which they designated pst, and which was selected on the basis of arsenate resistance (Bennett and Malamy, 1970). For the Pit system, one gene pit, mapping at 76 min, has been described (Sprague et al., 1975).

The claims of Willsky et al. (1973) that the inducible transport systems for α-glycerophosphate (GlpT), (Cozzarelli et al., 1968) and glucose-6-phosphate (Uhp), (Kornberg and Smith, 1969) were also involved in transporting Pi as a secondary

substrate have not been substantiated. Neither system can transport sufficient Pi to sustain the growth of strains lacking both the Pit and Pst systems (Sprague et al., 1975).

Strains of E. coli carrying either the Pst or the Pit system were constructed (Rosenberg et al., 1977; Willsky and Malamy, 1980) to facilitate their independent investigation. Rosenberg et al. (1977) found the Pst system to be inducible, while the Pit system was constitutive. The induction of the Pst system depended on protein synthesis. The comparison of kinetic parameters showed the transport constant (Kt) for the Pit system (25 μm) to be one hundred times the corresponding value of the Pst system (0.16 μm), while the maximal velocity of Pi uptake was the same in both systems. Rosenberg et al. (1977) also found that the two systems appeared to interact with a common intracellular Pi pool. This finding clearly indicated that the control of alkaline phosphatase synthesis is not a function of the intracellular concentration of Pi, since phoT mutants produce the enzyme constitutively in large amounts (Willsky et al., 1973), although their intracellular Pi pool is kept at normal levels by the operation of the Pit system. The role of the phosphate-binding protein in the Pst system was demonstrated by a comparison of Pi uptake in spheroplasts prepared from the Pit^+Pst^- and Pst^+Pit^- strains. The spheroplasts of the Pit^+Pst^- strain retained the ability to transport Pi but those of the Pst^+Pit^- strain did not. Finally, reconstitution of Pi uptake in spheroplasts of the Pst^+Pit^- strain with the phosphate-binding protein established unequivocally its role in the Pst system (Gerdes et al., 1977).

Willsky and Malamy (1980) reported that neither the Pst nor the Pit system exchanged internal ^{32}Pi for unlabelled Pi added externally. This contrasted with the observations of Rosenberg *et al.* (1977, 1982). This controversy was finally resolved when the GlpT system was shown to be responsible for mediating this exchange (Elvin *et al.*, 1985).

1.4B ENERGY COUPLING TO THE PIT AND PST SYSTEMS

In their comparison of the two systems, Rosenberg *et al.* (1977) found that, at pH6.9 and with glucose as the energy source, CCCP inhibited the Pit system by about 95% and the Pst system by about 12%. Membrane vesicles prepared from a Pit^+Pst^- strain transported Pi in the presence of oxidative (e.g. ascorbate and PMS) sources of energy (Konings and Rosenberg, 1978). This respiration-linked active transport was almost completely inhibited by CCCP. When membrane vesicles were prepared from a Pst^+Pit^- strain, active transport of Pi was not detected. In a more detailed examination (Rosenberg *et al.*, 1979) Pi uptake was studied in a set of strains carrying the Pit or Pst system alone in a normal, or ATPase-deficient background. These studies confirmed the absolute requirement of the Δp for the energization of the Pit system and of a phosphate-bond energy source for Pst. Under both aerobic and anaerobic conditions, either energy source could serve both systems when energy interchange was possible through an operative ATPase. In the strains with a defective ATPase only the appropriate form of energy, directly available, supported transport. CCCP abolished transport in the Pit system under all conditions, and abolished

it in the Pst system when lactate or succinate, but not glucose, provided the source of energy. Thus the Pit and Pst systems exhibit modes of energization characteristic of shock-resistant and shock-sensitive systems, respectively (Berger and Heppel, 1974). The effect of pH on initial rates of phosphate transport in E. coli in the presence and absence of CCCP was reported with the two systems by Rosenberg et al. (1984). In the pH range 5.5 to 7.8, glucose-energized cells carrying the Pst system exhibited a steady rate of uptake at all pH values, while succinate-energized cells showed nearly a 50% reduction of uptake at the higher pH. On the other hand, in cells carrying the Pit system, with either glucose or succinate as the energy source, the rate of phosphate uptake declined to about 30% from a maximum at pH6.0 as the pH rose to 7.8 ($\Delta pH=0$). Uptake in the Pit system was extremely sensitive to CCCP under most experimental conditions, while that in the Pst system was only partly affected. Following the report (Hong et al., 1979) that acetylphosphate was a likely candidate as the source of phosphate-bond energy in shock-sensitive transport, Rosenberg et al. (1984) checked the effect on Pi uptake of the insertion, into strains carrying the Pit or Pst system, of ack and pta (mutants carrying these alleles are unable to synthesize acetylphosphate). Although the loss of acetylphosphate affected the Pst system 3-fold more than the Pit, the complexity of the metabolic reactions involved ruled out the unambiguous identification of acetylphosphate as the energy source for the Pst system.

Studies on cation circulation associated with phosphate transport (Russell and Rosenberg, 1979,1980) provide an

explanation for the dependence of the Pst system on ΔpH (Rosenberg et al., 1984). Uptake of Pi is seen initially to be accompanied by a parallel uptake of protons. Pi uptake has an absolute requirement for the presence of K^+, and vice versa (Russell and Rosenberg, 1979). The link between Pi and K^+ transport is permissive, i.e. there is no direct link between any pair of potassium and phosphate transport systems (Russell and Rosenberg, 1980). The results were taken to indicate that Pi enters in symport with protons, the latter compensating for the import of two negative charges borne on the phosphate anion. The ensuing acidification of the cell's interior prompts an immediate expulsion of protons in exchange for K^+ ions.

1.4C RECENT GENETIC STUDIES AND THE IDENTIFICATION OF COMPONENTS OF THE PHOSPHATE-SPECIFIC TRANSPORT SYSTEM

1.4C1 THE PST SYSTEM OF ESCHERICHIA COLI

The alkaline phosphatase-constitutive mutants isolated by Garen and Otsuji (1964), while they fell into several groups defined by certain criteria, were in reality a great deal more complex. The two groups which carried mutations in the pst region, phoS and phoT (originally R2a and R2b, respectively) were classified by the observation that, while each resulted in the constitutive synthesis of alkaline phosphatase, only phoT mutants had the intact phosphate-binding protein which was lacking from the phoS mutants. In addition, another mutation pst, phenotypically similar to phoT, but considered distinct because it led to a lower level of alkaline phosphatase constitutivity,

was described (Willsky *et al.*, 1973). This was later referred to as *pst-1* and a similar allele, described by Sprague *et al.* (1975), became known as *pst-2*. Cox *et al.* (1981) identified, on the basis of complementation studies, another allele which they designated originally *pst-401*. They showed it to be in a complementation group which was distinct from either *phoT32* or *pst-2*, and proposed the renaming of *pst-2* as *pstA2*, and of *pst-401* as *pstB401*. Strains carrying the *pstB401* allele were constitutive for alkaline phosphatase and were unable to transport Pi through the Pst system.

Zuckier and Torriani (1981) reported that one of the *phoT* alleles (*phoT35*) belonged in a different complementation group from *phoS25* and *pst-2*. On the other hand Levitz *et al.* (1981) failed to observe complementation, assayed as a loss of alkaline phosphatase constitutivity, between all combinations of a pair of *phoS* alleles (*phoS21* and *phoS28*) and a pair of *phoT* alleles (*phoT9* and *phoT32*). These workers concluded that the *phoS* and *phoT* mutations were affecting the same cistron. Brucker *et al.* (1984) confirmed this observation, using the failure to restore Pi uptake as a criterion. Amemura *et al.* (1982) confirmed that *phoT35* was in a complementation group distinct from *phoS*, *phoT* or *pst-2*, and proposed for it the designation *phoU*.

1.4C2 A PHOSPHATE-SPECIFIC PORE IN THE OUTER MEMBRANE?

The first report of a possible role for the outer membrane in the complex phosphate-procuring process in *E. coli* came from Overbeeke and Lugtenberg (1980) who found that one of the several

outer membrane porins, the PhoE protein, was under Pi control. The structural gene (*phoE*) for this protein was located by Tommassen and Lugtenberg (1981) at 6 min on the *E. coli* chromosome. Korteland *et al.* (1982) found that the PhoE protein pore, in comparison with the OmpF and OmpC pores, was efficient for the passage of Pi and particularly so for organic phosphates. In this study the $PhoE^+$ strain transported Pi at a rate 9 times that observed in the $PhoE^-$ strain. That the pore is probably not essential for the transport of Pi, especially at high Pi concentration, is obvious from the normal operation of the Pit system under conditions where the synthesis of PhoE protein is repressed. The PhoE pore was tentatively considered to operate as a generalized anionic pore (Overbeeke and Lugtenberg, 1982), with a high specificity for various phosphate esters, and, especially, polyphosphate. While the function of the PhoE protein as a general anionic pore was later confirmed, it was still found to be the most efficient channel for Pi, particularly at low Pi concentrations (Korteland *et al.*, 1984). When tested in proteoliposomes, the PhoE pore proved far more effective in the passage of anions bearing multiple negative charges when compared to the OmpC and OmpF porins (Nikaido and Rosenberg, 1983).

1.5 THE *pho* REGULON OF *ESCHERICHIA COLI* K12

The *pho* (or phosphate) regulon of *E. coli* is composed of a set of regulatory and non-regulatory genes (see Tommassen and Lugtenberg, 1982; Wanner, 1983). The latter encode proteins that function in the acquisition of phosphate to meet the needs of the organism. Towards the fulfilment of this need there evolved a

complex system of gene regulation based primarily on the level of Pi in the extracellular medium. The first indication that regulation of gene expression could be mediated by the level of extracellular Pi was provided nearly 26 years ago. Horiuchi *et al.* (1959) and Torriani (1960) reported that when inorganic phosphate in the extracellular medium became growth-limiting, the synthesis of a phosphomonoesterase activity, named alkaline phosphatase, was induced. Mutations leading to the constitutive expression of alkaline phosphatase under normally repressing levels of Pi were subsequently mapped within two distinct regions of the *E. coli* chromosome, R1 and R2 (Echols *et al.*, 1961). The R2 region has since been shown to encode the high-affinity Pst system (see Sections 1.4A and 1.4C).

Mutations mapping in the R1 region could be assigned to three groups according to the phenotypic expression of alkaline phosphatase. The *phoR1a* phenotype is constitutive, but not fully derepressible by Pi limitation, while the *phoR1b* phenotype is constitutive and fully derepressible for alkaline phosphatase expression (Garen and Echols, 1962a). The third type of mutation, *phoR1c*, led to a phosphatase-negative phenotype (Garen and Echols, 1962b). Bracha and Yagil (1973) reported the identification of another gene, *phoB* (they originally named it *phoT*), which when mutated led to the loss of expression of alkaline phosphatase. A positive role in the expression of alkaline phosphatase was confirmed for the *phoB* gene product (Brickman and Beckwith, 1975; Yagil *et al.*, 1975). Kreuzer *et al.* (1975) reported that the *phoRa* and the *phoRb* (by this time the "1" had been dropped in the designation for complementation

groups in the R1 region) mutations were allelic, while the phoRc allele represented a different complementation group. The phoB and phoRc alleles were finally shown to represent mutations affecting the same cistron, when they were found not to complement in trans (Pratt and Torriani, 1977).

The phoB gene has since been cloned and the PhoB protein identified (Tommassen et al., 1982; Makino et al., 1982). Tommassen et al. (1982) also reported the cloning of the phoR gene and the identification of its gene product. The regulation of the phoB gene has been studied using a phoB-lacZ fusion (Shinagawa et al., 1983) and a phoB-cat fusion (Guan et al., 1983). The regulation of expression of the phoB gene was found by both groups to be very similar to that of phoA, the structural gene for alkaline phosphatase. It was subsequently shown (Makino et al., 1985) that the phoR gene was regulated in a similar manner to the phoB gene, and the authors concluded that both genes comprised an operon with the promoter located proximal to phoB. Increased transcription of the phoB-phoR operon occurred as a result of Pi limitation.

Wanner and Latterell (1980) described another gene, which they designated phoM, that had a positive role in the regulation of phoA expression. This gene was mapped at 0 min on the E. coli chromosome (Wanner and Bernstein, 1982). The phoM phenotype was found to be masked in a $phoR^+$ background. The residual expression of alkaline phosphatase present in a phoRa mutant has been attributed to the function of the PhoM protein (Wanner and Latterell, 1980; Wanner, 1983). The phoM gene has been cloned and its gene product identified (Makino et al., 1984; Tommassen

et al., 1984; Ludtke et al., 1985). This gene was found not to be regulated by the level of extracellular Pi (Makino et al., 1984; Ludtke et al., 1985).

The pleiotropic nature of mutations in the regulatory genes of the pho regulon was initially reported by Morris et al. (1974). These authors reported the constitutive synthesis of alkaline phosphatase and three other periplasmic proteins, P2, P3 and P4, in phoR, phoS and phoT mutants. Introduction of a phoB mutation abolished the synthesis of these proteins. P4 was subsequently shown to consist of two proteins, one of which is the phosphate-binding protein, the product of the phoS gene (Willsky and Malamy, 1976). Argast and Boos (1979) identified P2 as a binding protein for sn-glycerol-3-phosphate. It has not been possible to visualize P3 consistently, and it remains unidentified. An outer membrane porin, the PhoE protein (Tommassen and Lugtenberg, 1980), and the shock-sensitive ugp-dependent sn-glycerol-3-phosphate transport system (Schweizer and Boos, 1985), were also found to be subject to the same complex regulation as alkaline phosphatase. Furthermore it appears that there are several previously unidentified genes which are regulated by the level of Pi outside the cell. Using Mudl-directed lacZ fusions, Wanner and McSharry (1982) identified 15 separate phosphate-starvation-inducible (psi) promoters in E. coli K12. These psi promoters did not correspond to known Pi-regulated genes. Some of the psi promoters were shown to be induced by physiological triggers other than Pi starvation (Wanner and McSharry, 1982; Wanner, 1983). Thus some psi promoters respond to carbon and nitrogen deprivation, to

anaerobiosis and UV irradiation, and combinations thereof. It would therefore appear that the pho regulon may be part of an even larger global regulatory network consisting of overlapping and separate controls of gene expression.

1.6 THE MOLECULAR ARCHITECTURE OF SHOCK-SENSITIVE TRANSPORT SYSTEMS

The shock-sensitive active transport systems of gram-negative bacteria have several features in common (see Section 1.3C2). It is becoming evident that this similarity in function at the supramolecular level is reflected at the molecular level. The two shock-sensitive transport systems that have been well defined, the histidine permease of S. typhimurium (Higgins et al., 1982b) and the maltose transport system of E. coli (see Hengge and Boos, 1983), have a similar number of protein components. These proteins are located in the three fractions of the cell envelope: the outer membrane, the periplasmic space and the inner membrane.

1.6A COMPONENTS IN THE OUTER MEMBRANE

The outer membrane of gram-negative bacteria is an effective permeability barrier (see Lugtenberg and van Alphen, 1983; Nikaido and Vaara, 1985). Proteins which form pores, the so-called porins, facilitate the diffusion of solutes across this barrier. These porins can either be specific, or non-specific. The non-specific or "general" porins function in the diffusion of a range of solutes across the outer membrane. In E. coli, the

OmpF and OmpC porins facilitate the penetration of amino acids and sugars of a small size (<200 daltons) across the outer membrane (Pugsley and Schnaitman, 1978).

Unlike the general porins, the specific porins participate mainly in the active transport of a particular solute or group of related solutes. The best example of a specific porin is the LamB protein, a component of the maltose transport system of E. coli. LamB mutants do not take up maltotriose and have an increased Km^{app} for the transport of maltose (Szmelcman et al., 1976). The authors concluded that the LamB protein is essential for the diffusion of higher maltodextrins across the outer membrane, and that maltose diffusion through the outer membrane is the rate-limiting step in maltose uptake.

The PhoE protein, while not a specific pore for the permeation of Pi across the outer membrane, has nevertheless been shown to be the most efficient pore for diffusion of large anionic solutes (see Section 1.4C2).

1.6B COMPONENTS IN THE PERIPLASMIC SPACE

All shock-sensitive transport systems have a single water-soluble protein in the periplasmic space. These proteins, which are the most abundant component (about 40,000 molecules per cell) of shock-sensitive transport systems, bind their substrate in a very specific manner with high affinity (see Hengge and Boos, 1983). The binding proteins are released from the periplasmic space by a cold osmotic shock and by conversion of whole cells to

spheroplasts (Neu and Heppel, 1965), or membrane vesicles (Kaback, 1971). The uptake of solutes by shock-sensitive systems was impaired after release of the binding proteins (see Heppel, 1969) (hence the alternative designation of "binding protein-dependent" for "shock-sensitive" transport systems).

The first indication that periplasmic binding proteins were directly involved in the active transport of solutes came when these proteins were purified and found to possess many of the properties characteristic of cellular transport systems (see Wilson and Smith, 1978). It was assumed that these proteins were conferring on the transport systems their particular features. In this manner binding proteins have been characterized for the leucine (Penrose et al., 1968), phosphate (Medveczky and Rosenberg, 1970), glutamine (Weiner and Heppel, 1971), lysine (Rosen, 1971), cystine (Berger and Heppel, 1972), galactose (Boos, 1969), maltose (Kellerman and Szmelcman, 1974) and ribose (Willis and Furlong, 1974) transport systems of E. coli, and the sulphate (Pardee et al., 1966) and histidine (Ames and Lever, 1972) transport systems of S. typhimurium. Genetic evidence linking periplasmic binding proteins with solute transport has been presented for the galactose (Boos and Sarvas, 1970) and sn-glycerol-3-phosphate (Argast and Boos, 1979) transport systems of E. coli, and the histidine permease of S. typhimurium (Ames and Lever, 1970). Conclusive evidence for the role of binding proteins in shock-sensitive active transport was provided by the demonstration that addition of the respective purified binding protein to spheroplasts (Rosenberg et al., 1977) and membrane vesicles (Hunt and Hong, 1981) reconstituted active transport in

each case. The initial demonstration of reconstitution of active transport by the addition of purified binding protein to shocked cells (Anraku, 1968) has not been repeated in other laboratories (e.g. see Rae et al., 1976). However a generally applicable method for reconstitution of binding-protein dependent transport in mutants lacking the binding protein has been reported (Brass et al., 1983; Bukau et al., 1985). This method involved the addition of purified binding protein to cells which had been sequentially pretreated with Pi and $CaCl_2$.

Binding proteins have at least two specific sites which facilitate the role of these proteins in active transport (see Hengge and Boos, 1983). The first site is involved in the binding of substrate, while the second site is involved in the interaction of the binding protein with the inner membrane components (see Section 1.6C). These two sites were shown to be functionally distinct by Ames and Spudich (1976) who isolated a mutant, defective in histidine transport, which produced a histidine-binding protein that had an altered electrophoretic mobility but normal substrate-binding properties. Suppressor mutations, located in another gene, hisP, which encoded an inner membrane protein (Ames and Nikaido, 1978) of the transport system, were isolated which restored the transport of histidine. The authors concluded that the suppressor mutation allowed the inner membrane protein to "recognize" the mutant histidine-binding protein. Hunt and Hong (1983b) reported that the glutamine-binding protein could be selectively modified in a way that inhibited its ability to restore transport in membrane vesicles without altering the glutamine-binding properties of the

protein. Bavoil *et al.* (1983) have described a *malE* mutant of the maltose transport system which produces a maltose-binding protein (MBP) with a defect which abolishes the transport of maltose and maltodextrins, even though its substrate-binding properties were unaffected. This mutant was shown to possess a binding protein that was defective in the ability to interact with the LamB protein. Similarly, mutationally altered LamB proteins with a decreased ability to bind to MBP immobilized on a column have been reported (Luckey and Nikaido, 1983).

The nucleotide sequence of the genes encoding the histidine (*hisJ*) and lysine-arginine-ornithine (*argTr*) binding proteins of *S. typhimurium* (Higgins and Ames, 1981), and the ribose (Groarke *et al.*, 1983), leucine-isoleucine-valine and leucine-specific (Landick and Oxender, 1985) binding proteins of *E. coli*, and the amino acid sequence of the galactose-binding protein of *E. coli* (Mahoney *et al.*, 1983), have been reported. Recent structural analysis of periplasmic binding proteins by X-ray crystallography (Mowbray and Petsko, 1983; Quiocho and Vyas, 1984; Pflugrath and Quiocho, 1985) has shown that these proteins are composed of two globular domains. A substrate-binding cleft is located between the two domains. When the substrate is bound, the cleft is closed and no longer accessible to solvent.

Periplasmic binding proteins have an important role to play in bacterial chemotaxis, besides their role in active transport. The function of periplasmic binding proteins in the detection of extracellular stimuli, in the process of chemoreception, has been reviewed (Hazelbauer and Parkinson, 1977).

1.6C COMPONENTS IN THE INNER MEMBRANE

Pardee et al. (1966) reported that mutants defective in cystine transport still possessed a binding activity for cystine that could be released from the periplasmic space. The authors suggested that there were other components, besides the binding protein, that were involved in shock-sensitive transport. The first inner membrane protein of a shock-sensitive transport system to be identified on gel electrophoretograms was the HisP protein (Ames and Nikaido, 1978), a component of the histidine permease of S. typhimurium. The other inner membrane proteins of this system were discovered when the complete nucleotide sequence of the histidine transport operon was reported by Higgins et al. (1982b). Four genes, hisJ, hisQ, hisM and hisP, were identified. The HisQ protein was shown to be associated with the inner membrane. A similar localization has since been demonstrated for the HisM protein (Ames et al., 1985).

The maltose transport system of E. coli is another shock-sensitive transport system that is biochemically and genetically well defined. Five genes, malK, lamB, malE, malF and malG, have been found to be essential for the transport of maltose (Raibaud et al., 1979; Silhavy et al., 1979). The MalF protein (Shuman et al., 1980) and the MalK protein (Bavoil et al., 1980; Shuman and Silhavy, 1981) have been shown to be associated with the inner membrane. Shuman and Silhavy (1981) discovered that in a malG mutant most of the MalK protein could be recovered in the cytoplasmic fraction. This indicated that the MalK protein is localized to the inner membrane in wild strains by interaction

with the MalG protein. The malF (Froshauer and Beckwith, 1984) and the malG (Dassa and Hofnung, 1985) genes have been sequenced. The deduced amino acid sequence of both gene products indicates that they are hydrophobic.

The nucleotide sequence of the malK gene was reported (Gilson et al., 1982b) and found to share significant homology with the gene (ndh) encoding the respiratory NADH dehydrogenase. Sequence homology between the malK and the hisP genes was also found (Gilson et al., 1982a), but no consensus sequence could be described for the ndh, hisP and malK genes. In contrast Higgins et al. (1985) identified amino acid sequences corresponding to the consensus adenine nucleotide-binding fold (Walker et al., 1982) within the HisP, MalK, and OppD proteins (the latter is a component of the shock-sensitive oligopeptide permease of S. typhimurium). A functional ATP-binding site has been demonstrated for the HisP (Hobson et al., 1984) and the OppD proteins (Higgins et al., 1985).

Preliminary characterization of the mgl-dependent galactose transport system of S. typhimurium (Muller et al., 1985) and the high-affinity branched-chain amino acid permease of E. coli (Nazos et al., 1985) has indicated the presence of three inner membrane components in each system, while the ribose permease (Iida et al., 1984; Lopilato et al., 1984) and the mgl transport system (Harayama et al., 1983) of E. coli may contain two.

Two models have been proposed for the mechanism of shock-sensitive active transport (Higgins et al., 1982b). In the first model ("pore" model) the inner membrane proteins provide a non-

specific pore, while in the second model ("binding-site" model) one or more of the inner membrane proteins provides a binding site. The available evidence favours the binding-site model. Higgins et al. (1982b) described hisQ and hisM mutants which had a different substrate specificity compared to the wild-type transport system. Shuman (1982) constructed a strain with a non-polar deletion within the malE gene and isolated a revertant, which had regained the ability to transport maltose, but which could no longer transport maltodextrins. This transport, although independent of MBP, showed many of the characteristics of the wild-type transport system. It was subsequently shown (Treptow and Shuman, 1985) that maltose transport in such revertants resulted from mutations located in the malF and malG genes. The authors suggested that in the revertants a substrate-binding site on the inner membrane proteins, which is normally only accessible to substrate bound to MBP, is exposed allowing "free" substrate to bind.

1.7 AIMS

In view of the possible similarities between shock-sensitive transport systems, it was of interest to define the Pst system in more detail at the molecular level. In so doing information on the role of the Pst system in the regulation of expression of alkaline phosphatase and the other components of the pho regulon would also be obtained.

The experimental approach used to characterize the Pst system would involve:

i) DNA sequencing, and the identification of possible open reading frames (ORFs),

ii) relating individual ORFs to known genetic complementation groups,

iii) identifying the proteins encoded by the ORFs,

iv) genetic manipulation of the individual ORFs, and assessment of their effects on the transport and regulatory function of the Pst system,

v) establishing the nature of the regulation of the pst genes.

CHAPTER 2

MATERIALS AND METHODS

2.1 MATERIALS

Wherever possible, chemicals of analytical grade were used. Tetracycline, chloramphenicol, ethidium bromide, agarose, lysozyme and protease (Type VII) were purchased from Sigma Chemical Co., St. Louis, Mo., U.S.A. Spectinomycin hydrochloride (Trobicin) was obtained from Upjohn Co., Kalamazoo, U.S.A. and ampicillin from Beecham Research Laboratories, Victoria, Australia. Ampholines were purchased from LKB Produkter AB, Bromma, Sweden. Restriction endonucleases, T4-DNA ligase, carrier-free [^{32}P]orthophosphate, [α-^{32}P]deoxyadenosine triphosphate, [α-^{32}P]deoxycytidine triphosphate, [γ-^{32}P]adenosine triphosphate, and L-[^{35}S]-methionine were from Amersham Australia, Pty. Ltd. DNA polymerase I (large fragment, Klenow enzyme), T4 polynucleotide kinase, nuclease Bal31 and calf intestinal alkaline phosphatase were obtained from Boehringer Mannheim, GmbH, W. Germany. Agar, tryptone, yeast extract and casamino acids were purchased from Difco, Detroit, Michigan, U.S.A. Peptone was obtained from Oxoid Ltd., England.

2.2 MEDIA

All media were sterilised by autoclaving at 121°C for 30min, or where appropriate, by filtration.

2.2A GROWTH SUPPLEMENTS

All growth supplements, prepared as concentrated solutions, and sterilised separately, were added to sterile rich or minimal medium as required.

Glucose (20mM), sodium succinate (20mM), or sodium lactate (25mM) were used as carbon source at the concentrations indicated unless otherwise specified. N-acetyl-D-glucosamine was used at 20mM as a carbon source, and at 0.9mM otherwise. Other supplements were used at the following final concentrations:

Thiamine hydrochloride	1μM
L-arginine	0.7mM
L-isoleucine	0.32mM
L-valine	0.36mM
L-leucine	0.65mM
L-threonine	0.70mM
L-histidine	0.21mM
adenine	0.1mM
2,3-dihydroxy benzoate (DHB)	30μM
uracil	0.2mM
casein hydrolysate	0.05% (w/v)
biotin	2-4nM
thymine	0.002% (w/v)

The following antibiotics were used:

tetracycline	10μg/ml
ampicillin	50μg/ml

chloramphenicol	100µg/ml
cycloserine	100µg/ml

2.2B LURIA BROTH

Luria broth, described by Luria and Burrous (1957), contained (per litre):

Tryptone	10g
Yeast extract	5g
sodium chloride	10g

The pH was adjusted to 7.0 with sodium hydroxide prior to autoclaving.

2.2C LURIA GLYCEROL

Luria-glycerol, used for the storage of bacterial strains, contained a 100:30 (v/w) mixture of Luria broth and glycerol.

2.2D HIGH PHOSPHATE MINIMAL MEDIUM

The high phosphate minimal medium used for the growth of strains was similar to that described by Monod et al. (1951) as medium 56, and contained:

K_2HPO_4	61mM
NaH_2PO_4	39mM
$(NH_4)_2SO_4$	15mM
$MgSO_4$	0.8mM

This was prepared as a stock solution, which included all the components except magnesium sulphate, at twenty times the above concentration and stored under a 1:1 mixture of chloroform and toluene. The concentrated medium was diluted to single strength and supplemented, before autoclaving, with magnesium sulphate and a trace metal concentrate (Gibson et al., 1977b) to a final concentration of:

$FeCl_3.6H_2O$	1.78μM
$CaCl_2.2H_2O$	2.45μM
$MnSO_4.H_2O$	1.0μM
$ZnSO_4.7H_2O$	13.9μM
$CoSO_4.7H_2O$	0.68μM
H_3BO_3	4.69μM

This was made up as an 1000-fold concentrate and added to the salts medium before sterilisation.

2.2E LOW PHOSPHATE MINIMAL MEDIUM

This medium was similar to that described by Gerdes and Rosenberg (1974). It contained:

Tris	50mM
KCl	10mM
$(NH_4)_2SO_4$	10mM
$MgSO_4$	0.8mM
Yeast extract	0.1% (w/v)

A metal ion supplement was also used as described in Section 2.2D. The pH was adjusted to 7.4 with HCl prior to sterilisation.

2.2F NUTRIENT MEDIA

The nutrient medium used throughout this study (referred to as 'rich medium') contained, per litre:

Beef extract	10.0g
Peptone	10.0g
Yeast extract	3.0g
NaCl	5.0g
Glucose	2.0g

The pH was adjusted to 7.5 with NaOH before sterilisation.

Z medium, used solely for the propagation of bacteriophage, contained:

Tryptone	1% (w/v)
Yeast extract	0.1% (w/v)
NaCl	137mM
$CaCl_2$	2.5mM
Glucose	5mM

The pH was adjusted to 7.0 with NaOH before sterilisation.

2.2G PHOSPHATE-FREE MEDIUM

This medium, used to deplete cells of Pi prior to Pi uptake, and for Pi uptake measurement, contained:

MOPSO	25mM
KCl	40mM
$(NH_4)_2SO_4$	10mM
$MgSO_4$	1mM

The pH was adjusted to 6.5 with KOH.

2.2H WASH SOLUTION

This solution was used for washing the filtration membranes during ^{32}Pi uptake measurements. It contained:

Triethanolamine	10mM
KCl	100mM
$(NH_4)_2SO_4$	10mM
$MgSO_4$	1mM

The pH was adjusted to 6.5 with HCl.

2.2I SOLID MEDIA

Solid media were prepared by adding 2% (w/v) agar to the medium required. In the preparation of rich medium, the agar was mixed with the rich medium prior to autoclaving. For minimal

medium equal volumes of double-strength media and 4% (w/v) aqueous agar were mixed after autoclaving. Sterile supplements were added where appropriate before pouring.

2.3 BACTERIAL STRAINS AND PLASMIDS

Details of the bacterial strains used are described in Table 2.1. The plasmids used in this study are listed in Table 2.2. Strains were stored in Luria-glycerol at -20°C.

2.3A GROWTH MEASUREMENTS IN LIQUID MEDIA

Turbidity of cell cultures was used as a measure of cell density. Cultures were measured against a blank of the same medium, using a Klett-Summerson colorimeter fitted with a blue filter. Cell density was expressed in arbitrary Klett units where 10^8 cells/ml is approximately equivalent to a reading of Klett 40.

Cell densities were also measured on a Gilford 300 spectrophotometer at 595nm or 660nm. The measurements at 660nm were converted to mg dry weight of cells per ml, using the experimentally derived factor:

$$A_{660} \text{ of } 1.0 = 0.43\text{mg dry weight/ml}$$

2.3B GROWTH OF BACTERIA

For large scale culture cells were grown, unless otherwise

Table 2.1. Strains of *E. coli* K12 used in this study.

STRAIN	RELEVANT GENOTYPE	NOTES OR REFERENCE
AN346	*ilvC argH pyrE entA*	Gibson *et al.* (1977a)
AN1403	*unc-449 pstB401 argH pyrE entA srl*::Tn*10 recA*	Cox *et al.* (1981)
AN1416	pAN26/*unc-449 pstB401 argH pyrE entA srl*::Tn*10 recA*	Partial diploid strain with an F-prime plasmid carrying the *pst* region from strain AN1403 constructed as described in Chapter 2 (Section 2.9A).
AN1459	*ilvC thr leu srl*::Tn*10 recA*	Laboratory stock
AN1664	*ilvC argH entA srl*::Tn*10 recA*	Laboratory stock
AN1667	pAN92/*ilvC argH entA srl*::Tn*10 recA*	Cox *et al.* (1981)
AN1685	*pstA2 argH pyrE entA srl*::Tn*10 recA*	Strain AN1664 carrying plasmid pAN92 (Chapter 4)
AN1696	*phoT32 argH pyrE entA srl*::Tn*10 recA*	Cox *et al.* (1981)

.../contd. ...

Table 2.1. (/contd.) Strains of *E. coli* K12 used in this study.

STRAIN	RELEVANT GENOTYPE	NOTES OR REFERENCE
AN1700	*pstA34 argH pyrE entA srl*::Tn*10* *recA*	Laboratory stock. The *pstA34* allele was previously designated *phoT34* (see Chapter 4)
AN1706	pAN115/*ilvC argH pyrE purE* nal^r *recA*	Strain with an F-prime plasmid, carrying the *pstA34* allele, constructed as described in Chapter 2 (Section 2.9A). Laboratory stock
AN2098	pAN127/*pstA2 argH pyrE entA srl*::Tn*10* *recA*	Obtained by transformation of strain AN1685 with pAN127 (Chapter 3)
AN2197	pAN263/*unc-449 pstB401 argH pyrE entA srl*::Tn*10* *recA*	Isolated after transformation of strain AN1403 with pAN263 (Chapter 4)
AN2199	pAN263/*pstA2 argH pyrE entA srl*::Tn*10* *recA*	Isolated after transformation of strain AN1685 with pAN263 (Chapter 4)
AN2200	pAN263/*phoT32 argH pyrE entA srl*::Tn*10* *recA*	Isolated after transformation of strain AN1696 with pAN263 (Chapter 4)

.../contd. ...

Table 2.1. (/contd.) Strains of *E. coli* K12 used in this study.

STRAIN	RELEVANT GENOTYPE	NOTES OR REFERENCE
AN2221	*phoU35 trpR rpsL srl::Tn10 recA*	Isolated after transduction of strain WC4 with strain NK5304 as donor
AN2222	pAN271/*phoU35 trpR rpsL srl::Tn10 recA*	Isolated after transformation of strain AN2221 with pAN271 (Chapter 4)
AN2223	pAN272/*phoU35 trpR rpsL srl::Tn10 recA*	Isolated after transformation of strain AN2221 with pAN272 (Chapter 3)
AN2224	pAN127/*unc-449 pstB401 argH pyrE entA srl::Tn10 recA*	Isolated after transformation of strain AN1403 with pAN127 (Chapter 3)
AN2254	pAN271/*unc-449 pstB401 argH pyrE entA srl::Tn10 recA*	Isolated after transformation of strain AN1403 with pAN271 (Chapter 4)
AN2255	pAN271/*pstA2 argH pyrE entA srl::Tn10 recA*	Isolated after transformation of strain AN1685 with pAN271 (Chapter 4)
AN2256	pAN271/*phoT32 argH pyrE entA srl::Tn10 recA*	Isolated after transformation of strain AN1696 with pAN271 (Chapter 4)

.../contd. ...

Table 2.1. (/contd.) Strains of *E. coli* K12 used in this study.

STRAIN	RELEVANT GENOTYPE	NOTES OR REFERENCE
AN2257	pAN127/*phoT32 argH pyrE entA srl*::Tn*10 recA*	Isolated after transformation of strain AN1696 with pAN127 (Chapter 3)
AN2258	pAN127/*phoU35 trpR rpsL srl*::Tn*10 recA*	Isolated after transformation of strain AN2221 with pAN127 (Chapter 3)
AN2259	pAN263/*phoU35 trpR rpsL srl*::Tn*10 recA*	Isolated after transformation of strain AN2221 with pAN263 (Chapter 4)
AN2260	pAN272/*unc-449 pstB401 argH pyrE entA srl*::Tn*10 recA*	Isolated after transformation of strain AN1403 with pAN272 (Chapter 3)
AN2261	pAN272/*pstA2 argH pyrE entA srl*::Tn*10 recA*	Isolated after transformation of strain AN1685 with pAN272 (Chapter 3)
AN2262	pAN272/*phoT32 argH pyrE entA srl*::Tn*10 recA*	Isolated after transformation of strain AN1696 with pAN272 (Chapter 3)
AN2351	*lacZ53 rpsL51 thyA36 polA12*^{ts} *rha-5 deoC2* λ^- *rrnD-rrnE*	Obtained from the Coli Genetic Stock Centre (CGSC, New Haven, Conn., USA)

.../contd. ...

Table 2.1. (/contd.) Strains of E. coli K12 used in this study.

STRAIN	RELEVANT GENOTYPE	NOTES OR REFERENCE
AN2537	ΔpstC345 argH pyrE entA	Isolated after transduction of the ΔpstC345 allele into strain AN346 (Chapter 5)
AN2538	ΔpstC345 argH pyrE entA srl::Tn10 recA	Isolated after transduction of strain AN2537 with strain NK5304 as donor
AN2539	argH pyrE entA	Isogenic sibling of strain AN2537 (Chapter 5)
AN2564	pAN115/ΔpstC345 argH pyrE entA srl::Tn10 recA	Isolated by mating strain AN1706 with strain AN2538 (Chapter 5)
AN2565	pAN26/ΔpstC345 argH pyrE entA srl::Tn10 recA	Isolated by mating strain AN1416 with strain AN2538 (Chapter 5)
AN2566	pAN357/ΔpstC345 argH pyrE entA srl::Tn10 recA	Isolated after transformation of strain AN2538 with pAN357 (Chapter 5)
AN2568	pAN115/pstA34 argH pyrE entA srl::Tn10 recA	Partial diploid strain carrying the pst region from strain AN1700. Isolated as described in Chapter 2 (Section 2.9A)

.../contd. ...

Table 2.1. (/contd.) Strains of *E. coli* K12 used in this study.

STRAIN	RELEVANT GENOTYPE	NOTES OR REFERENCE
DL538	*trpR rpsL* Δ(*tna-glmS*)	Obtained from A. Torriani (Massachusetts Institute of Technology)
HR299	*trpR rpsL* Δ(*tna-glmS*) *srl*::Tn*10* *recA*	Isolated after transduction of strain DL538 with strain NK5304 as donor (Chapter 6)
HR300	pAN356/*trpR rpsL* Δ(*tna-glmS*) *srl*::Tn*10* *recA*	Isolated after transformation of strain HR299 with pAN356 (Chapter 6)
HR301	pAN357/*trpR rpsL* Δ(*tna-glmS*) *srl*::Tn*10* *recA*	Isolated after transformation of strain HR299 with pAN357 (Chapter 6)
HR303	pAN275/*his bio* λ^{def} *cI857*	Isolated after transformation of strain N4830 with pAN275 (Chapter 7)
HR304	pAN276/*his bio* λ^{def} *cI857*	Isolated after transformation of strain N4830 with pAN276 (Chapter 7)
N4830	*his bio* λ^{def} *cI857*	Gottesman *et al.* (1980)
NK5304	*srl-1300*::Tn*10* *recA56*	Cox *et al.* (1981)

Genetic nomenclature from Bachmann (1983).

Table 2.2. Plasmids used in this study.

PLASMID	RELEVANT GENETIC LOCI	DERIVATION OR REFERENCE
pACYC184	*cat tet*	Chang and Cohen (1978)
pAD329	*amp N*	Derivative of plasmid pBR322 obtained from N. Dixon
pAN26	*pyrE*$^+$ *phoS*$^+$*pstC*$^+$*A*$^+$*B401phoU*$^+$ *ilvC*$^+$ *argH*$^+$	F-prime plasmid carrying *pstB401* allele, obtained as described in Chapter 2 (Section 2.9A). Laboratory stock
pAN36	*cat phoS*$^+$*pstC*$^+$*A*$^+$*B*$^+$*phoU*$^+$	Downie *et al.* (1980)
pAN45	*cat phoS*$^+$*pstC*$^+$*A*$^+$*B*$^+$*phoU*$^+$	Downie *et al.* (1980)
pAN92	*cat phoS*$^+$*pstC*$^+$*A*$^+$*B*$^+$*phoU*$^+$	Jans *et al.* (1983)
pAN115	*pyrE*$^+$ *phoS*$^+$*pstC*$^+$*A34B*$^+$*phoU*$^+$ *ilvC*$^+$ *argH*$^+$	F-prime plasmid carrying *pstA34* allele, obtained as as described above for pAN26. Laboratory stock
pAN127	*cat pstA*$^+$*B*$^+$*phoU*$^+$	Cox *et al.* (1981)
pAN263	*cat pstA*$^+$	This study - Chapter 4 (see Fig. 4.1)
pAN271	*cat phoU*$^+$	This study - Chapter 4 (see Fig. 4.1)
pAN272	*cat*	This study - Chapter 3 (see Fig. 3.2)
pAN274	*amp*	This study - Chapter 7 (see Fig. 7.1)

.../contd. ...

Table 2.2. (/contd.) Plasmids used in this study.

PLASMID	RELEVANT GENETIC LOCI	DERIVATION OR REFERENCE
pAN275	amp $pstB^+phoU^+$	This study - Chapter 7 (see Fig. 7.1)
pAN276	amp $pstB^+phoU^+$	This study - Chapter 7 (see Fig. 7.1)
pAN346	cat $phoS^+\Delta pstC345pstA^+B^+phoU^+$	This study - Chapter 5 (see Fig. 5.1)
pAN354	cat $pstA^+pstB^+\Delta phoU1$	This study - Chapter 6 (see Fig. 6.1)
pAN355	cat $phoS^+pstC^+A^+B^+\Delta phoU1$	This study - Chapter 6 (see Fig. 6.1)
pAN356	amp $phoS^+pstC^+A^+B^+phoU^+$	This study - Chapter 6 (see Fig. 6.4)
pAN357	amp $phoS^+pstC^+A^+B^+\Delta phoU1$	This study - Chapter 6 (see Fig. 6.4)
pMF3	amp	Manis and Kline (1977)

Genetic nomenclature derived from Novick et al. (1976), and Bachmann (1983).

stated, in the high phosphate minimal medium (supplemented with casamino acids, 0.05% w/v) or in the low phosphate minimal medium containing a carbon source and other growth requirements. Plates of rich medium were inoculated from storage (Section 2.3), and one plate used to inoculate a litre of growth medium. Cells were grown in a 14 litre New Brunswick fermenter in 10-litre batches inoculated with one litre of a 16h culture. All cells were grown at 37°C, and batches were harvested after about 16h by centrifugation in a Sharples centrifuge.

Small scale cultures were grown in Erlenmeyer flasks of appropriate size filled to 25-30% capacity and shaken at 37°C. In the growth and preparation of bacteria for ^{32}Pi uptake studies, cells (50ml cultures) were grown overnight (16h), harvested by centrifugation, washed twice with the phosphate-free medium containing a carbon source and other supplements as required, and suspended in the same medium at A_{660} of 0.35. Except where otherwise stated, the carbon source used was sodium lactate (25mM). The cells were then shaken for 2h at 37°C to deplete them of Pi and to induce maximal uptake in the Pst system.

The cells were then harvested, washed twice in the uptake medium containing the necessary supplements and resuspended in the same medium at A_{660} of 0.35. Cells were stored for up to an hour until required for uptake assays. All procedures were carried out at room temperature.

2.3C PREPARATION OF SUBCELLULAR FRACTIONS

Membranes were prepared as described by Cox et al. (1973) and Senior et al. (1979). Briefly, washed cells were suspended in STEM buffer containing 40mM ε-amino-n-caproic acid (EACA) and 6mM ρ-aminobenzamidine (PAB) using 2ml/g wet weight of cells. STEM buffer contained:

250mM Sucrose
100mM 2-{(2-hydroxy-1,1-bis(hydroxymethyl)ethyl)amino}-ethanesulphonic acid, (TES), pH7.0
0.25mM ethyleneglycol tetraacetic acid (EGTA)
20mM magnesium acetate.

The cells were disintegrated in a RIBI cell fractionator operated at 137MPa. Cell debris was removed by centrifugation at 25000xg for 0.5h. The supernatant was centrifuged at 254000xg for 2h. The pellet, containing the membrane fraction, was resuspended in STEM buffer in the presence of the proteinase inhibitors EACA (40mM) and PAB (6mM). The supernatant ("periplasmic-cytoplasmic" fraction) was retained if required.

The membranes were washed three times in low-ionic strength buffers. The buffer system described above and a buffer system containing TES (50mM), glycerol (15%, v/v), EACA (40mM) and PAB (6mM) was used in a 1:4 ratio (v/v) in the first wash. In the subsequent washes the buffer system used, "5mM TES", contained TES (5mM), glycerol (15%, v/v), EACA (40mM) and PAB (6mM). The washed membrane preparation was resuspended in the "5mM TES" buffer system.

Membranes were occasionally washed once with the "5mM TES" buffer system containing salt (1M sodium phosphate or 5M NaCl, see Chapter 4). This wash was performed in addition to the washes described above. Solutions containing proteins extracted from the membrane were concentrated by ultrafiltration and dialysed overnight against "5mM TES" buffer, before electrophoretic analysis. All procedures were carried out at 4°C.

2.3D ANALYSIS OF PROTEIN COMPOSITION OF WHOLE CELLS

Cells from a 10ml culture at a cell density at 595nm of about 1.5 were chilled on ice and harvested by centrifugation at 7000xg for 15min at 0°C. The pellets were resuspended to a cell density at 595nm of about 100 in a solution containing 25% (w/v) sucrose and 50mM Tris, pH8.0. A sample of the cell suspension (1µl) was diluted with 9µl of the above solution and mixed with 10µl of a solution containing 0.54M Tris base, 67mM DTT, 2% (w/v) SDS, 0.08% (w/v) bromophenol blue, and 20% (w/v) glycerol ("cracking buffer"). The cells were then lysed by heating the mixture at 100°C for 2min, and the lysates subjected to electrophoresis on one-dimensional gels containing a separating gel with a uniform concentration of acrylamide (Section 2.5D)

2.4 BIOCHEMICAL ASSAYS

2.4A ASSAY OF ALKALINE PHOSPHATASE ACTIVITY

The alkaline phosphatase activity of periplasmic-cytoplasmic

fractions was assayed quantitatively as described by Cox et al. (1981). The reaction mixture contained:

160mM Tris-HCl (pH9.0)
1mM zinc acetate
20mM ρ-nitrophenyl phosphate

in a final volume of 3ml. After pre-incubation at 30°C the reaction was started by the addition of the relevant extract, usually 30-50µg protein/ml of reaction mixture. The reaction was stopped at 1min intervals by transferring 0.5ml of reaction mixture to 4.5ml of 2M NaOH. The absorbance was measured at 410nm, and the amount of ρ-nitrophenol released was determined from its molar absorbance, 1.62×10^4 $M^{-1}cm^{-1}$.

Strains with the constitutive phenotype of alkaline phosphatase demonstrated detectable alkaline phosphatase activity (>0.5µmol ρ-nitrophenol formed/min per mg protein) following growth in the presence of high levels of extracellular Pi. Conversely, strains with the phosphate-repressible phenotype had lower levels of alkaline phosphatase activity (<0.1µmol ρ-nitrophenol formed/min per mg protein) when grown under the same conditions.

For a rapid determination of the alkaline phosphatase activity of bacterial strains the method of Bracha and Yagil (1969) was used. Bacterial colonies were transferred from a plate onto Whatman filter discs (12.5cm) by replica plating. The discs were sprayed with a 1:1 (v/v) mixture of α-naphthyl phosphate (2mg/ml in 1M Tris-HCl, pH8.0) and tetrazotized o-

dianisidine (10mg/ml in water). The two reagents were dissolved separately and mixed just before use. With this spray the presence and absence of alkaline phosphatase activity was indicated by a purple and a yellow colouration, respectively.

2.4B MEASUREMENT OF ^{32}Pi UPTAKE

The cell suspensions at A_{660} 0.35, prepared as in 2.3B, were diluted 1 in 25 in the same medium (uptake medium with a carbon source and other supplements required) and were shaken in a water bath at 37°C for 4min. Uptake was initiated by adding ^{32}Pi to a final concentration of 0.5µM and a specific radioactivity of about 8GBq/mmol. Samples (0.5ml) of cell suspension were withdrawn at the appropriate times and filtered through cellulose nitrate membranes (pore size 0.45µm), using a filtration apparatus described by Rosenberg (1979). The filters were washed with two 2ml portions of the wash solution, removed from the filtration apparatus and pressed upside-down onto stainless steel planchets. The dried filters were counted in a Nuclear Chicago gas-flow counter.

Standards for specific radioactivity and blanks (filtrations without cells) were counted at the same time. Uptake was expressed as nmol substrate/min per mg dry weight of cells.

2.4C PROTEIN DETERMINATION

Protein concentrations were determined using Folin's phenol reagent (Lowry *et al.*, 1951), or by the protein-dye binding

method (Bradford, 1976), with bovine serum albumin (fraction V; Sigma Chemical Co., St. Louis, Mo.) as a standard.

2.5 GEL ELECTROPHORESIS

2.5A ACRYLAMIDE GEL ELECTROPHORESIS OF DNA

Acrylamide gels used for the electrophoresis of DNA were prepared from either a 30% or a 40% (w/v) stock solution. The latter contained 38% acrylamide and 2% bisacrylamide, while the former contained 29% acrylamide and 1% bisacrylamide. Acrylamide gels were run vertically on an apparatus of appropriate size, with TBE buffer (90mM Tris-borate, pH8.3, 3mM EDTA) as the running buffer.

Analysis of restriction endonuclease digests of plasmid DNA was carried out on 8% acrylamide slab gels. These gels were 90mm high by 150mm long by 1.5mm thick, and contained wells 15mm deep by 7mm wide for the loading of samples. Each gel contained TBE buffer (see above) and 8% (w/v) acrylamide (from the 30% stock solution) in a final volume of 30ml, and was polymerized by the addition of 160µl of ammonium persulphate (10%, w/v) and 80µl of Temed. The gel was run for 2.5h at 23mA constant current at room temperature, and then stained for 20min in a solution of ethidium bromide (1µg/ml), visualized under UV light and photographed through a red filter.

For preparation of restriction fragments, digests of plasmid DNA were electrophoresed on 8% acrylamide slab gels (90mm high by

150mm long by 3mm thick, with wells each 15mm deep and up to 120mm wide for the loading of samples). The gel consisted of TBE buffer and 8% (w/v) acrylamide (see above) in a final volume of 50ml and was polymerized by the addition of 300µl of ammonium persulphate (10%, w/v) and 150µl of Temed. Electrophoresis was performed at a constant current of 30mA for 3 to 6h, depending on the size of the restriction fragments to be separated. The gel was stained as above, but with a solution of 0.5µg/ml ethidium bromide, and visualized under UV light.

Separation of the ^{32}P-labelled ends of DNA fragments was performed on 6% or 8% acrylamide gels (175mm wide by 365mm long by 0.8mm thick, containing wells 2cm deep and 1.5cm wide for the loading of samples). The gel contained TBE buffer (see above) and 6% or 8% (w/v) acrylamide (from the 30% stock solution) in a volume of 60ml, and was polymerized by adding 240µl of ammonium persulphate (10%, w/v) and 120µl of Temed. 8% gels (used for DNA strand separation after melting) were run at a constant voltage of 300V for 16-20h, while 6% gels (used for separation of fragments following enzymatic scission) were run for 2-5h at a constant current of 30mA. In each case electrophoresis was carried out at room temperature over a period of time considered necessary to optimize the separation of the labelled ends.

Gels used in the DNA sequencing procedure were 20% or 6% (w/v) acrylamide slab gels (175mm wide by 365mm long by 0.35mm thick, containing wells 12mm deep and 3mm wide for the loading of samples). The gels consisted of TBE buffer, 50% (w/v) urea, and acrylamide at the appropriate concentration (from the 40% w/v

stock solution) in a volume of 40ml. Gels of 20% acrylamide were polymerized by the addition of 75µl of ammonium persulphate (10%, w/v) and 50µl of Temed, and 6% gels by adding 130µl of ammonium persulphate and 65µl of Temed. Gels were run at a constant power of 20 Watts, for 3h in the case of 20% gels, and for times varying from 2 to 8h in the case of 6% gels. Staggering the loading of samples on the 6% gels allowed different regions of overlapping sequence to be obtained, thus enabling the complete sequence of long DNA fragments (up to 300 nucleotides) to be determined in one operation.

2.5B AGAROSE GEL ELECTROPHORESIS OF DNA

The agarose gels were 155mm long and 155mm wide, and contained wells 3-6mm deep and 5mm wide for the loading of samples. Agarose gels (0.6 or 0.8%, w/v) were made up in a buffer containing 40mM Tris-acetate, pH7.8, 20mM sodium acetate and 10mM EDTA (TEA buffer), and were run at 1-1.5V/cm for 16h. Gels were stained, visualized and photographed as above.

2.5C TWO-DIMENSIONAL GEL ELECTROPHORESIS OF PROTEINS

Membrane suspensions, derived as in Section 2.3C, were extracted three times with five volumes of cold acetone to remove lipids. During each extraction the membrane-acetone mixture was incubated for 5min on ice, and centrifuged at room temperature. The final pellet was spread around the inside of the tube, and dried under reduced pressure. The protein was suspended in approximately 0.5ml of lysis buffer (9.5M urea, 2% w/v Nonidet P-

40, 2% w/v ampholines [1.2% pH range 5-7 and 0.8% pH range 3.5-10], and 5% v/v 2-mercaptoethanol) prior to electrophoresis.

Periplasmic-cytoplasmic fractions, and solutions of proteins extracted from the membrane fraction (see Section 2.3C) were mixed with an equal volume of lysis buffer (see above) before electrophoresis.

Two-dimensional gel electrophoresis was carried out using the system of O'Farrell (O'Farrell, 1975; O'Farrell et al., 1977).

In the first dimension, isoelectric focusing, polypeptides were separated on the basis of their isoelectric point. The cylindrical gels (5mm diameter by 130mm) consisted of 9.2M urea, 2% (w/v) Nonidet P-40, 4% (w/v) acrylamide/bisacrylamide (from a 30% w/v stock solution comprising 28.4% acrylamide and 1.6% bisacrylamide) and ampholines with a pH range 5-7 (1.2%, w/v) and a pH range 3.5-10 (0.8%, w/v). The mixture was polymerized by the addition of 10µl of ammonium persulphate (10%, w/v) and 7µl Temed per 10ml. The gel was then overlayed with 8M urea. Samples (100-300µl) were loaded and subjected to electrophoresis for 4h starting at 180V and maintaining constant current until the voltage reached 800 (about 3h), which was then maintained for the remainder of the run. The cathode solution (20mM NaOH) was in the bottom reservoir and the anode solution (10mM phosphoric acid) in the top reservoir. After electrophoresis the gels were placed in SDS sample buffer (2% SDS, 125mM Tris-HCl, pH6.8) and stored at -20°C.

In the second dimension the polypeptides were separated on the basis of size in a linear polyacrylamide gradient containing SDS (SDS-PAGE). The slab gel (90mm high by 150mm long by 1.5mm thick) consisted of a stacking gel and a separating gel. The separating gel contained a gradient of acrylamide and glycerol and was poured first by mixing two solutions in a gradient mixer with two chambers. The front chamber contained 350mM Tris-HCl, pH8.8, 0.01% (w/v) SDS, 11.7% glycerol (v/v) and 23% (w/v) acrylamide (from a 30% w/v stock solution containing 29.2% acrylamide and 0.8% bisacrylamide) in a volume of 11.75ml. Polymerization was initiated by the addition of 15µl 10% (w/v) ammonium persulphate and 3µl of Temed. The back chamber contained 350mM Tris-HCl, pH8.8, 0.01% (w/v) SDS, and 9.7% (w/v) acrylamide (from the above 30% stock solution) in a volume of 11.75ml, and was polymerized by the addition of 25µl 10% (w/v) ammonium persulphate and 5µl of Temed. The stacking gel (14ml) was 4.5% (w/v) acrylamide (from the above 30% stock solution), 0.1% (w/v) SDS and 125mM Tris-HCl, pH6.8. Polymerization was initiated by the addition of 42µl of 10% (w/v) ammonium persulphate and 14µl of Temed. The slab gel was then assembled onto the running apparatus and the cylindrical gel from the first dimension was placed across the top of the stacking gel and secured in place with 1% (w/v) agarose. Running buffer (25mM Tris base, 192mM glycine and 0.1% w/v SDS) was added to each reservoir and electrophoresis performed for 16h at 12mA constant current.

After electrophoresis the gel slab was immersed in a solution containing 5.5% (w/v) trichloroacetic acid, 5.5% (w/v)

sulphosalicylic acid and 11% (v/v) methanol and kept at 65°C for 60min in order to fix the proteins. The gel was then stained with 0.11% (w/v) Coomassie Blue G-250 in 25% (v/v) ethanol and 8% (v/v) acetic acid for 60min, and destained for 1-2h in the above solvent. It was stained a second time for 60min, and after exhaustive destaining proteins were readily visualized.

2.5D ONE-DIMENSIONAL GEL ELECTROPHORESIS OF PROTEINS

The one-dimensional slab gels used for the electrophoretic separation of proteins consisted of a stacking gel and a separating gel, which contained either a linear polyacrylamide gradient or a uniform concentration of acrylamide. One-dimensional gels consisting of a polyacrylamide gradient in the separating gel were equivalent to the second dimension of the two-dimensional gels (Section 2.5C). A comb was inserted into the stacking gel prior to polymerization to obtain wells 15mm deep and 7mm wide for the loading of samples. The gels were run for 17h at a constant current of 8mA. After electrophoresis the gels were fixed, stained and destained as described for the two-dimensional gels (Section 2.5C).

One-dimensional gels consisting of a separating gel with a uniform concentration of acrylamide were 90mm high by 150mm long by 0.5mm thick, with wells 15mm deep and 4mm wide for the loading of samples. The separating gel contained 12% (w/v) acrylamide (from a stock solution containing 30% (w/v) acrylamide and 0.8% (w/v) bisacrylamide), 0.375mM Tris, pH8.8, and 0.1% (w/v) SDS, in a final volume of 15ml. Polymerization was initiated by adding

50µl of 10% (w/v) ammonium persulphate and 5µl of Temed. The stacking gel contained 5% (w/v) acrylamide (from the above stock solution), 125mM Tris, pH6.8, and 0.1% (w/v) SDS, in a final volume of 5ml, and was polymerized with 40µl of 10% (w/v) ammonium persulphate and 4µl of Temed. The gel was run at 60V constant voltage, for electrophoresis of proteins through the stacking gel, and at 120V constant voltage for electrophoresis through the separating gel. The running buffer contained 50mM Tris base, 384mM glycine and 0.1% (w/v) SDS. After electrophoresis the gel slab was fixed and stained at room temperature in a solution containing 0.25% (w/v) Coomassie Blue G-250, 45% (v/v) methanol and 9% (v/v) acetic acid. The gel was destained as described above for two-dimensional gels.

2.6 DNA MANIPULATIONS

2.6A PREPARATION OF DNA

Amplifiable plasmid DNA was isolated by the method of Selker et al. (1977). Plasmid-bearing strains were grown at 37°C in 1 litre batches of high phosphate minimal medium supplemented with 0.5% (w/v) casamino acids, 40mM glucose and other growth requirements, and an appropriate antibiotic to select for plasmid retention. The growth was monitored by measuring the turbidity of the culture, and spectinomycin (300mg/litre) was added when the cell density was 90-100 Klett. Cultures were grown for a further 12-16h for optimal plasmid amplification.

All the operations from this stage were carried out at 4°C. The cells were pelleted by centrifugation at 6000xg for 10min

(in a Sorvall RC-2B Centrifuge with a GS3 rotor) and resuspended in 20ml of a solution containing 25% (w/v) sucrose and 50mM Tris, pH8.0. Lysozyme (40mg), and after 5min, 4ml of 0.25M EDTA, were added. After a further 5min, 32ml of Triton buffer (3% w/v Triton X-100, 50mM Tris, pH8.0, 62.5mM EDTA) was added. After 15min the lysate was centrifuged at 35000xg for 20min at 4°C (in a Sorvall RC-2B centrifuge with an SS-34 rotor). To the supernatant ("cleared lysate"), NaCl (5M) and polyethylene glycol 6000 (50%, w/v) were added to final concentrations of 1M and 10% (w/v), respectively. Precipitation was allowed to occur over 2-18h, whereupon the precipitate was harvested by centrifugation at 6000xg for 5min (Sorvall RC-2B centrifuge and GSA rotor).

The pellet was resuspended in TE buffer (10mM Tris, pH8.0, 1mM EDTA) and treated with 200µl of protease (Type VII from Bacillus amyloliquefaciens, 20mg/ml in TE, previously autodigested at 80°C for 10min) for 1h at 37°C. The mixture (7.2ml) was added to 7.4g of caesium chloride. The solution was centrifuged at 10400xg for 30min at 15°C (Sorvall RC-2B centrifuge and SE-12 rotor), and the supernatant transferred to a polyallomer ultracentrifuge tube (Beckman). Ethidium bromide (0.8ml of a 10mg/ml solution) was added to the tube, which was then capped, topped up with liquid paraffin, and centrifuged at 150000xg_{av} for 43h at 15°C in a Beckman L8-70 Ultracentrifuge with a fixed-angle 50Ti or 75Ti rotor.

After centrifugation, the plasmid DNA band was visualized under UV light and drawn from the tube with a sterile disposable 1ml syringe. Ethidium bromide was removed by three extractions

with NaCl-saturated isopropanol (stored over TE). The aqueous layer was retained and dialysed overnight at 4°C against 1.5 litres of TE diluted ten-fold.

MiniF plasmid DNA was isolated by the method described above except that strains bearing miniF plasmids were grown without amplification and harvested when the cell density was about 220 Klett.

Chromosomal DNA was isolated by a scaled-down Triton-lysis procedure. The cells from 500ml of culture (grown without amplification) were harvested by centrifugation at 4°C and resuspended in 2.5ml of a solution containing 25% (w/v) sucrose, 50mM Tris, pH8.0, 62.5mM EDTA, and 20mg of lysozyme. After 5min on ice, 1ml of 0.5M EDTA was added. After a further 5min, 3ml of Triton buffer was added and the solution evenly mixed, prior to incubation for 30min on ice. Protease (0.5ml of a 100mg/ml solution) was added, and the mixture incubated for 1h at 37°C. The lysate (6.3ml) was then added to 5.85g of caesium chloride and 0.63ml of ethidium bromide (10mg/ml). Density gradient centrifugation, and subsequent extraction of the chromosomal DNA from the tube, were as described above. Prior to restriction, the chromosomal DNA was routinely subjected to phenol and then ether extractions.

2.6B PRECIPITATION OF DNA

DNA was precipitated from aqueous solution in 270µl aliquots in Eppendorf microfuge tubes, to which 30µl of 3M sodium acetate

and 1ml of absolute ethanol were added. The contents were vortexed and the tubes were kept for 5min in dry ice/ethanol (-70°C), and then centrifuged for 5min at 4°C in an Eppendorf Microfuge (12500rpm). The supernatant was discarded and 1ml of 70% (v/v) ethanol containing 0.1mM EDTA was added to each tube, without resuspending the pellet. The tubes were cooled at -70°C for 5min and then centrifuged for 5min. The pellets were finally washed, as above, with 1ml of absolute ethanol. The pellets were dried under reduced pressure, and then stored at -20°C until required.

2.6C RESTRICTION OF DNA

DNA (0.5-150µg) was digested in the buffers listed in Table 2.3. Ten-fold concentrates of each buffer were prepared and sterilised by autoclaving or filtration, where appropriate. Reaction mixtures were incubated for 4-6h at 37°C with sufficient restriction endonuclease to completely digest the DNA. Alternatively, the mixtures were incubated overnight. Digestions with PstI were carried out at 30°C, with BstEII at 60°C and with TaqI at 65°C.

2.6D DIGESTION OF DNA WITH NUCLEASE BAL31

The DNA was linearized by digestion with a restriction endonuclease at a unique site. The reaction mixture was then extracted once with one volume of phenol and twice with two volumes of ether, retaining the aqueous layer at each step. The DNA was precipitated (see Section 2.6B) and the dried pellet

Table 2.3. Restriction endonuclease buffers.

ENZYME	Tris-HCl (mM) and pH	$MgCl_2$ (mM)	NaCl (mM)	KCl (mM)	2-mercapto-ethanol (mM)
AluI	6, 7.6	6	-	-	6
AvaI	6, 7.4	6	100	-	6
BstEII	100, 7.6	5	-	-	-
DpnI, and PvuI	10, 7.4	6	150	-	6
EcoRI	100, 7.5	5	50	-	2
HaeII, HaeIII and HhaI	50, 7.5	5	-	-	0.5
HindIII	20, 7.4	7	60	-	-
HinfI	6, 7.5	6	100	-	-
HpaI	20, 7.4	10	-	6	1
HpaII	10, 7.4	10	-	6	1
MluI	10, 7.5	7	100	-	7
PstI	6, 7.4	6	50	-	6
RsaI	6, 8.0	12	50	-	6
Sau3A	6, 7.5	15	60	-	6
TaqI	10, 8.4	6	-	-	6

resuspended to a final volume of 100µl in a solution containing 20mM Tris-HCl (pH8.0), 12mM $CaCl_2$, 200mM NaCl and 1mM EDTA. The DNA solution was preincubated at 25°C for 3min, and the nuclease Bal31 was then added in a ratio of 1U:5µg DNA. Ten-µl portions of the reaction mixture were removed at 0, 1, 2, 5, 10, 15, 20 and 30min, and added to Eppendorf microfuge tubes containing EGTA (2mM, final concentration) to quench the reaction. To each mixture was added 1/3 volume of sample buffer (14.5% w/v sucrose, 0.045% w/v bromophenol blue, 50mM EDTA) prior to electrophoresis on agarose gels (Section 2.6I) and purification of DNA fragments (Section 2.6J).

2.6E PHOSPHATASE TREATMENT OF DNA

To remove the terminal phosphates of DNA fragments prior to ligation 0.1U of calf intestinal alkaline phosphatase (CIP) was added to reaction mixtures containing DNA and restriction endonuclease. In the phosphatase treatment of DNA prior to [γ-^{32}P]ATP end-labelling, DNA fragments (25-50µg) purified as in Section 2.8A were resuspended in a medium containing 50mM Tris-HCl (pH8.0) and 0.1mM EDTA, and treated with 5-10U of CIP. Phosphatase treatment was carried out at 37°C for 30min.

The CIP was then inactivated by extracting twice with an equal volume of phenol saturated with TBE buffer (Section 2.5A), and residual phenol was extracted twice with two volumes of ether. The aqueous layer containing the DNA was retained after each extraction. The DNA was precipitated as described in Section 2.6B prior to further use.

2.6F LIGATION OF DNA

Before ligation the DNA fragments in solution were occasionally treated with CIP (see Section 2.6E) to increase the possibility of obtaining a particular ligation event. On occasions when treatment with phosphatase was not required, the restriction endonucleases present in the DNA solution were inactivated by heating to 65°C for 10min, before ligation. The restriction fragments were mixed with ligation buffer, which consisted of 5µl of 1M Tris-HCl, pH8.0, 5µl of 130mM $MgCl_2$, 5µl of 2mM ATP and 5µl of 200mM dithiothreitol (DTT) per 100µl final reaction mixture. Ligation was performed for 12-20h at 12°C with 1U of T4-DNA ligase.

2.6G TRANSFORMATION OF PLASMID DNA AND LIGATION MIXTURES

Cells were transformed with DNA essentially as described by Lederberg and Cohen (1974). Cells were grown in 20ml of Luria broth at 37°C to a density of 100 Klett, chilled on ice, centrifuged at 3000xg for 10min at 4°C and resuspended in an equal volume of cold 10mM $MgSO_4$. Following a 30min incubation on ice the cells were centrifuged, resuspended in 10ml of cold 50mM $CaCl_2$, and kept on ice for a further 15min. After centrifugation at 4°C the cell pellet was resuspended in 1ml of cold $CaCl_2$. For transformation, 0.2ml of the calcium-treated cells was mixed with 100ng of DNA. The mixture was incubated on ice for 15min, heated for 2min at 42°C and then diluted into 5ml of Luria broth. After a 90min incubation to permit cell recovery and full expression of plasmid-encoded genes, aliquots were spread onto selective medium and incubated for 1 to 2 days at 37°C.

2.6H PLASMID SIZING PROCEDURE

A modified procedure of Barnes (1977) was used routinely to screen strains for the presence and size of recombinant plasmids present in multi-copy. The cells from a sector of growth on solid medium were vortexed in 40µl of TEA electrophoresis buffer (see Section 2.5B) and 70µl of a solution containing 5% (w/v) SDS, 50% (v/v) glycerol, 100mM EDTA and 0.2% (w/v) xylene cyanol, and lysed by heating at 70°C for 30min. The chromosomal DNA was then sheared by vigorous vortexing for 30sec and 100µl of the lysed cell suspension was electrophoresed on a 9mm thick agarose gel (0.8%, w/v) as described in Section 2.5B.

Strains were screened for the presence and size of recombinant plasmids present in unit-copy as follows. Plasmids were prepared from cells grown in 50ml cultures by an appropriately scaled-down Triton-lysis procedure (Section 2.6A), with the following modifications. After precipitation of DNA with NaCl and polyethylene glycol 6000, the DNA was resuspended in TE and the aqueous solution extracted three times with two volumes of phenol and three times with two volumes of ether. The DNA was then precipitated (Section 2.6B), resuspended in TE and treated with ribonuclease (Sigma Chemical Co., St. Louis, Mo.) at room temperature for 10min. The DNA samples were then mixed with 1/3 volume of sample buffer (Section 2.6D), and electrophoresed as described in Section 2.5B.

2.6I GEL ELECTROPHORESIS OF RESTRICTED DNA

Restriction endonuclease digests of plasmid DNA were analysed by gel electrophoresis. Digests containing large DNA fragments (>600 base pairs in size) were electrophoresed on 5mm thick, 0.6% or 0.8% (w/v) agarose gels (Section 2.5B), and digests containing smaller restriction fragments (50-800 base pairs in size) were electrophoresed on 8% acrylamide slab gels (Section 2.5A). Reaction mixtures containing DNA and restriction endonuclease were mixed with 1/3 volume of sample buffer (Section 2.6D), and about 30µl (equivalent to approx. 0.2µg DNA) was electrophoresed.

2.6J PURIFICATION OF DNA FROM AGAROSE GELS

DNA solutions containing sample buffer (Section 2.6D) were electrophoresed on a 0.6% (w/v) agarose gel as described in Section 2.5B. The gel and the running buffer contained 0.5µg/ml of ethidium bromide, and the DNA fragments in the gel were visualized under UV light. After the DNA fragments had migrated an appropriate distance into the gel, a strip of diethylaminoethyl-81 paper (which had been washed in 2.5M NaCl for 30min, rinsed twice with distilled water and washed in TEA buffer for 5min) was inserted into the gel ahead of the desired fragment. Electrophoresis was continued until most of the DNA had migrated into the paper. The paper was then removed from the gel, rinsed in distilled water and placed in an Eppendorf microfuge tube containing 600µl of 2M NaCl. The paper was macerated and the mixture was kept for 3h at 37°C. The paper

pulp was then separated from the NaCl solution, and the solution extracted twice with two volumes of iso-amyl alcohol, twice with two volumes of phenol (saturated with TBE buffer) and three times with two volumes of ether. The NaCl concentration of the aqueous layer was then adjusted to 1M, and the precipitation of DNA initiated by the addition of two volumes of absolute ethanol. Except for the method of salt precipitation of DNA employed here, the DNA was precipitated as described in Section 2.6B.

2.7 *IN VITRO* TRANSCRIPTION/TRANSLATION SYSTEM

Synthesis of proteins with covalently closed plasmid DNA as the template was accomplished by a modified form of the *in vitro* transcription/translation system of Zubay *et al.* (1971). The reaction mixture contained:

10µl Solution A
10µl Solution B
10µl Solution C
20µl [^{35}S]methionine (39.6TBq/mmol; 441MBq/ml)
20µl DNA (100µg, in 20mM Tris-acetate, pH7.9, 0.1mM EDTA)
10µl 2.2mM L-leucine
20µl S-30

The reaction was initiated by the addition of S-30, and was carried out for 30min at 34°C. It was terminated by the addition of 100µl of lysis buffer (see Section 2.5C). The mixture was frozen in liquid nitrogen and stored at -70°C until electrophoretic analysis.

Solution A contained 165µl of solution A1, and 50µl of solution A2. Solution A1 consisted of:

571mM Tris-acetate, pH8.2
714mM potassium acetate
337mM ammonium acetate
0.35mg/ml pyridoxine
0.14mg/ml PAB

Solution A2 was:

60mM DTT
9.4mM each of the following L-amino acids:

Glycine, alanine, valine, isoleucine, serine, threonine, aspartate, glutamate, lysine, histidine, arginine, phenylalanine, tyrosine, proline, asparagine, glutamine and cysteine.

Solution B was:

211mM trisodium-PEP
22mM ATP
5.5mM each of CTP, GTP, and UTP
1mg/ml *E. coli* tRNA
27mg/ml each of calcium-leucovorin, NADP, and FAD

Solution C was prepared by mixing equal volumes of a solution containing 0.14M magnesium acetate and 0.148M calcium acetate, and a solution of 2mM L-tryptophan. All solutions were stored

frozen at -20°C. This gave the following final concentrations for all reagents:

	µmoles/ml
Tris-acetate, pH8.2	44
DTT	1.4
potassium acetate	55
each amino acid	0.22
tryptophan	0.1
CTP, GTP, UTP	0.55
ATP	2.2
trisodium-PEP	21
ammonium acetate	27
magnesium acetate	12.8
calcium acetate	7.4
tRNA	100
pyridoxine-HCl	27
NADP	27
FAD	27
calcium leucovorin	27
PAB	11
DNA	0.0003 (approx.)
S-30	6000 (approx.)

The S-30 fraction was prepared from strain AN1720, a pho constitutive derivative of the rna strain PR7. Cells were harvested at a density of 200 Klett and washed twice with approximately 10 volumes Buffer A, which contained:

10mM Tris-acetate, pH8.2
14mM magnesium acetate
60mM potassium acetate
1mM DTT

The pellet (25-30g) was resuspended in 1.5 volumes of the same buffer, and the cells were broken at 137MPa in a Ribi cell fractionator. The extract was centrifuged at 30000xg for 30min. The supernatant was centrifuged again at 30000xg for 20min prior to a pre-incubation to remove endogenous mRNA (Nirenberg, 1963). This involved incubating 10ml of the supernatant with 2.46ml of the pre-incubation mixture at 37°C for 80min in the dark. The pre-incubation mixture contained:

406mM Tris-acetate, pH7.8
11.3mM magnesium acetate
0.04mM L-methionine
0.004 vol Solution A2
3.3mM ATP (pH7.0)
37.5mM trisodium-PEP
0.042mg/ml pyruvate kinase

After pre-incubation the extract was dialysed against Buffer A for 6-8h, and 0.5ml portions were frozen in liquid nitrogen and stored at -70°C.

The [^{35}S]methionine-labelled products of the *in vitro* transcription/translation system were analysed by electrophoresis on two-dimensional (Section 2.5C) or one-dimensional gradient

gels (Section 2.5D). After fixing, staining and destaining (see Section 2.5C) gels were dried under reduced pressure, and then exposed for 3 to 10 days to Kodak X-ray film, which was developed using a Kodak X-omat M20 Processor.

2.8 DNA SEQUENCING

2.8A PREPARATION OF RESTRICTION FRAGMENTS

To isolate DNA fragments for sequencing by the method of Maxam and Gilbert (1980), three aliquots of 100-150µg of plasmid DNA (isolated as in Section 2.6A) were incubated overnight with sufficient restriction endonuclease to effect complete digestion. The restriction endonucleases routinely used were TaqI and HinfI (buffer conditions as described in Section 2.6C, Table 2.3). Each digest was mixed with 1/3 volume of sample buffer (Section 2.6D), loaded onto a prerun 8% (w/v) acrylamide slab gel (see Section 2.5A), and electrophoresed. After staining and visualization, DNA fragments of the desired size were cut from the gel, and equivalent fragments from each gel pooled.

Gel pieces were placed in dialysis sacs containing about 0.5ml TBE buffer. The sacs were then placed in the same buffer in a flat bed gel apparatus, at right angles to the direction of current flow, and subjected to 300V constant voltage for 30min at room temperature, to electro-elute the DNA from the gel. The buffer was then removed from the sacs, and subjected to three extractions with two volumes of iso-amyl alcohol, to remove the ethidium bromide. This was followed by two extractions with two

volumes of distilled phenol (saturated with TBE buffer), and three extractions with two volumes of ether. The aqueous layer containing the DNA was retained at each step. The DNA was then precipitated as described in Section 2.6B.

2.8B [α-^{32}P]dATP AND [α-^{32}P]dCTP END-LABELLING

The procedure for [α-^{32}P]dATP end-labelling was as follows: aliquots (5-20µl) of [α-^{32}P]deoxyadenosine triphosphate (approx. 111TBq/mmol; 370MBq/ml) were freeze-dried in Eppendorf microfuge tubes. To each tube was added:

13µl DNA (purified restriction fragment from Section 2.8A resuspended to 13µl in water)
3µl 0.5mM deoxycytidine triphosphate
3µl 0.5mM deoxyguanosine triphosphate
3µl 0.5mM deoxythymidine triphosphate
6µl "cocktail"
1µl 0.75M DTT
1µl DNA polymerase I (Klenow fragment), 5U

"Cocktail" consisted of 130µl of 0.25M sodium phosphate, pH7.0, and 5µl of 1.0M $MgCl_2$, and was made up just before use.

The 30µl mixture was incubated for 30min at 37°C, and 3µl of 0.5mM un-labelled deoxyadenosine triphosphate was added as a chase. The mixture was incubated for a further 20min at 37°C, and then 240µl H_2O, 30µl 3M sodium acetate, and 1ml of absolute ethanol added. The DNA was precipitated as described in Section 2.6B, and the pellet dried under reduced pressure.

In the end-labelling of DNA fragments with [α-^{32}P]deoxycytidine triphosphate, an equivalent amount of [α-^{32}P]deoxycytidine triphosphate (approx. 111TBq/mmol; 370MBq/ml) was used as the source of radioactivity. The procedure used, including the volume and concentration of reagents, was similar to that employed for [α-^{32}P]dATP end-labelling, with the following exceptions. Non-radioactive deoxyadenosine triphosphate was used instead of non-radioactive deoxycytidine triphosphate in the end-labelling reaction with DNA polymerase I, while non-radioactive deoxycytidine triphosphate was used in the chase.

2.8C [γ-^{32}P]ATP END-LABELLING

The procedure used for [γ-^{32}P]ATP end-labelling of DNA fragments was essentially as described by Maxam and Gilbert (1980). Aliquots (5-20µl) of [γ-^{32}P]adenosine triphosphate (approx. 111TBq/mmol, 370MBq/ml) were freeze-dried in Fppendorf microfuge tubes. To each tube was added:

9µl DNA (purified restriction fragment from Section 2.8A resuspended to 9µl in H_2O)
35µl 20mM Tris-HCl, pH9.5, 1mM spermidine, 0.1mM EDTA
5µl 500mM Tris-HCl, pH9.5, 100mM $MgCl_2$, 50mM DTT, 50% (v/v) glycerol
1µl T4 polynucleotide kinase, 8U

The 50µl mixture was incubated for 1h at 37°C. The reaction was stopped, and the precipitation of DNA initiated, by the addition of 200µl 2.5M ammonium acetate, 1µl tRNA (1mg/ml), and 750µl of

absolute ethanol. After vortexing the mixture was kept for 5min in dry ice/ethanol (-70°C), and then centrifuged for 5min at 4°C in an Eppendorf Microfuge (12500rpm). The pellet of DNA was resuspended in 250µl of 0.3M sodium acetate and 750µl of absolute ethanol, kept at -70°C for 5min, and then centrifuged for 5min. Absolute ethanol (1ml) was then added to the tube, without resuspending the pellet, and the tube kept at -70°C for 5min, and then centrifuged for 5min. The pellet was dried under reduced pressure.

2.8D LABELLED-END SEPARATION

The two radioactively-labelled ends of the DNA fragment were separated either by strand separation (Maxam and Gilbert, 1980), or by digestion with a second restriction endonuclease. Strand separation was used mainly for labelled fragments of less than 200 base pairs in size. DNA was dissolved in 100µl of 30% (v/v) dimethylsulfoxide, 1mM EDTA, 0.05% (w/v) xylene cyanol and 0.05% (w/v) bromophenol blue. The mixture was heated at 90°C for 5min, and quickly chilled in ice water. Aliquots (25µl) of the samples were immediately loaded onto a pre-run 8% (w/v) acrylamide slab gel (see Section 2.5A).

DNA fragments greater in size than 200 base pairs were routinely cleaved with a second restriction endonuclease to separate the labelled ends. The DNA was dissolved in approximately 24µl of water and 3µl of the appropriate 10-fold buffer concentrate (Table 2.3), and a sufficient amount of the appropriate restriction endonuclease added to ensure maximal

digestion overnight. The sample, after addition of 1/4 volume of 10% (w/v) sucrose, 0.05% (w/v) xylene cyanol, and 0.05% (w/v) bromophenol blue, was loaded onto a pre-run 6% acrylamide slab gel (see Section 2.5A).

After electrophoresis the glass plates that enclosed the 6% or 8% gel were prised apart, leaving the gel lying on one plate. The gel was then covered with Saran wrap, exposed to Kodak X-ray film at room temperature for 3min, and the autoradiograph developed using a Kodak X-omat M20 Processor. Desired fragments were cut from the gel using the autoradiograph as a guide.

2.8E TREATMENT OF DNA PRIOR TO SEQUENCE REACTIONS

Labelled DNA was extracted from the acrylamide gel fragments by electro-elution as described in Section 2.8A. The DNA, in a volume of 270µl, was then precipitated with 3M sodium acetate and absolute ethanol, and the pellet washed with 70% ethanol containing 0.1mM EDTA, and with absolute ethanol, as in Section 2.6B. The pellet of DNA was dried under reduced pressure, and the dried pellet was resuspended in 32µl H_2O and 6µl of 1mg/ml sonicated calf thymus carrier DNA, prior to sequencing reactions.

2.8F SEQUENCING REACTIONS

The rationale of the enhanced cleavage at particular nucleotides is explained by Maxam and Gilbert (1977). The sequencing reactions were carried out with the following additions:

1. cytosine/thymine enhanced cleavage:

10µl of labelled DNA (+ carrier DNA)
10µl H_2O
10µl hydrazine

2. cytosine enhanced cleavage:

8µl labelled DNA (+ carrier)
15µl saturated NaCl
30µl hydrazine

3. adenine/guanine enhanced cleavage:

10µl labelled DNA (+ carrier)
25µl formic acid

4. guanine enhanced cleavage:

8µl labelled DNA (+ carrier)
200µl DMS buffer
1µl dimethylsulphate

DMS buffer contained 50mM sodium cacodylate, pH8.0, and 1mM EDTA.

The reactions were carried out at 20°C. Reaction 4 was stopped after 2min by the addition of 50µl of "G-stop" solution and 1ml of absolute ethanol, and subsequently kept at -70°C (dry ice/ethanol).

Reaction 3 was stopped after 4min by the addition of 250µl of "A,C,T-stop" solution and 1ml of absolute ethanol, and subsequently kept at -70°C. Reactions 1 and 2 were stopped after 5 and 6min, respectively, in identical fashion to Reaction 3.

"G-stop" solution contained 1.5M sodium acetate, pH7.0, 1.0M 2-mercaptoethanol, and 100µg/ml tRNA. "A,C,T-stop" solution contained 0.3M sodium acetate, pH7.0, 0.1mM EDTA and 25µg/ml tRNA.

All samples were cooled for at least 5min at -70°C, and then centrifuged for 5min on an Eppendorf Microfuge (12500rpm). The pellets were resuspended in 250µl of 0.3M sodium acetate, pH7.0, and 1ml of absolute ethanol, kept at -70°C for 5min and centrifuged for 5min. Absolute ethanol (1ml) was then added to each tube, without resuspending the pellet, and the tubes kept at -70°C for 5min and centrifuged for 5min. The pellets were dried under reduced pressure.

Each pellet was resuspended in 75µl of 9.9% (v/v) aqueous piperidine and heated at 90°C for 16min. H_2O (50µl) was added, the samples frozen at -70°C (dry ice/ethanol), and lyophilized. A further 50µl of H_2O was added, and lyophilization repeated. A final addition of 100µl of H_2O was made and the solution lyophilized overnight.

2.8G SEQUENCING GELS

The radioactivity of samples was determined by Cerenkov

radiation in a Packard Tri-Carb 300 counter. The samples were resuspended in 90% (v/v) deionised formamide containing 1mM EDTA, 0.05% (w/v) xylene cyanol and 0.05% (w/v) bromophenol blue, to a specific activity of $50x10^3$-$100x10^3$ cpm/µl. Samples were heated at 90°C for 5min to denature the DNA and immediately chilled in ice water. Each sample (1µl) was then electrophoresed on a pre-run 20% or 6% (w/v) acrylamide slab gel (see Section 2.5A).

After electrophoresis the gel was transferred to a sheet of used X-ray film and covered with Saran wrap. Gels were exposed for 8-36h at -70°C to Kodak X-ray film in a Kodak X-omatic C-2 Cassette, with intensifying screens whenever necessary. Films were developed as in Section 2.8D, and DNA sequence ladders read.

2.9 GENETIC MANIPULATIONS

2.9A CROSS-STREAKING TEST

These tests for genetic complementation were carried out with strain AN346 (*entA ilvC argH pyrE*) and its *ilv*$^+$ derivatives.

Episomes carrying *pst* alleles had been constructed previously (Cox *et al.*, 1981). Briefly, an F-prime plasmid, from which the region covering the *ilv* and *pst* genes had been deleted, was transferred into strains which were *ilv*$^+$ and *pst*$^-$. Recombination occurred as a rare event, resulting in the transfer of the *pst* alleles and the *ilv* genes from the chromosome onto the F-prime plasmid, thus repairing the deletion. The "repaired"

plasmid was transferred to a recA strain which also contained ilvC, pyrE, argH and purE mutations. This plasmid complemented the first three mutations and the purE marker was used to select against this strain when it was used as a male in genetic complementation studies. Subsequently this plasmid could be used to construct partial diploids covering the pst region in any recipient strain.

Donor strains (males), carrying the purE marker on the chromosome, were grown to a density of 100 Klett in a glucose-Luria broth at 37°C, centrifuged, and concentrated 10-fold in medium 56 containing Ca^{2+} (10^{-3}%, w/v) and Fe^{2+} ($5x10^{-5}$%, w/v). Recipient strains (females), which contained the entA, argH and pyrE mutations in addition to a pst allele, were grown and prepared in the same manner, but were not concentrated. The recA female strain was streaked along a plate containing 2,3-DHB and casamino acids, and the male strains subsequently streaked at right angles. Only female strains carrying an F-prime plasmid (partial diploids) grew on this medium, as the plasmid complemented the pyrE and the argH mutations and 2,3-DHB relieved the auxotrophy caused by the entA mutation.

The partial diploid strains carried a pst mutation on the chromosome and a pst mutation on the plasmid. These strains were purified from single colonies by re-streaking transconjugants on the same selective medium, and the alkaline phosphatase activity assayed qualitatively by the spray method (Section 2.4A).

2.9B BACTERIOPHAGE P1 TRANSDUCTION

The donor strain was grown in Luria broth at 30°C or 37°C to a cell density of 200 Klett. A sample (0.1ml) of cells, and 0.1ml of a fresh bacteriophage preparation, were added to a sterile test tube containing Z medium (Section 2.2F) and agar (0.8%, w/v). The contents of the tube were mixed gently by hand and poured onto the surface of a Z plate. After the soft agar overlay had set the plate was incubated for about 8h at 37°C. Sterile NaCl (3ml of 0.9%, w/v) was added to the plate, and the incubation continued for 2-3h at 4°C. The soft agar was mashed with the NaCl solution, and the mixture transferred to a sterile centrifuge tube. Chloroform (0.1ml) was added and the suspension vortexed vigorously before centrifugation at 17000xg for 5min on a bench centrifuge. The supernatant, which contained bacteriophage, was stored at 4°C in the presence of 0.1ml of chloroform.

The recipient strain to be transduced was grown at 37°C in Luria broth to a cell density of 250 Klett. A sample (0.1ml) of the culture was added to a test tube containing 0.1ml of bacteriophage suspension from the donor strain and $CaCl_2$ (5mM). The mixture was incubated without agitation at 37°C for 30min. Sodium citrate (to 77mM) was added and, after vortexing, samples were spread on agar plates containing the particular selective medium. Samples (0.1ml) of both the recipient strain culture and the bacteriophage suspension were spread on selective plates as controls. Transductants appeared on the selective plates as single colonies after incubation at 37°C for 1 to 2 days.

2.9C *recA* CONSTRUCTION

recA derivatives were isolated by transduction using as donor the strain NK5304, which carries the co-transducible alleles *recA* and *srl*::Tn*10*. Transduction was carried out in the normal manner, using P*1kc* grown on NK5304. Transductants were selected on rich medium containing tetracycline. Resistant colonies were screened for the presence of the *recA* allele by determining sensitivity to UV irradiation. This was performed by streaking a colony onto a plate of rich medium containing tetracycline and exposing the plate for 30sec to the short wavelength radiation of a Chromota-vue cabinet (Ultra-violet Products, San Gabriel, CA, USA). The *rec*$^+$ cells survived this exposure.

2.9D COMPLEMENTATION TESTS WITH RECOMBINANT PLASMIDS

pst mutant strains transformed with plasmid DNA were selected on rich medium (see Section 2.2F) containing ampicillin or chloramphenicol, depending on the antibiotic resistance marker carried on the vector component of the plasmid. 50-100 colonies were patched onto a plate of rich medium, and replicated onto plates of selective medium containing high phosphate (Section 2.2D), supplemented with the appropriate antibiotic. The alkaline phosphatase activity of these colonies was then tested by the spray method (Section 2.4A). When a quantitative estimate of alkaline phosphatase activity was required, a typical colony was purified and its alkaline phosphatase activity measured (Section 2.4A). Where appropriate the Pi uptake rate of the purified transformant was also determined (Section 2.4B).

Complementation of the chromosomal pst mutation by the plasmid was inferred when repression of alkaline phosphatase activity under conditions of high Pi concentration, and restoration of Pi transport activity (where appropriate), were observed.

2.9E TRANSFER OF PLASMID DNA TO THE CHROMOSOME

To integrate plasmid DNA into the chromosome, single colonies of chloramphenicol-resistant transformants of strain AN2351 carrying derivatives of the vector plasmid pACYC184 were inoculated into 10ml of Luria broth containing thymine (LBT) and chloramphenicol, and grown for 16h at 30°C. The stationary phase culture was serially diluted in 0.9% NaCl and samples plated on rich media containing chloramphenicol and incubated at 42°C for 18h.

Selection for segregation of plasmid DNA which had integrated into the chromosome was performed as follows. A single colony of a strain derived from AN2351 with plasmid integrated into the chromosome was grown in 10ml of LBT for 16h at 42°C. A 1ml sample of this culture was added to 50ml of LBT, and the cells were grown at 42°C to an optical density at 590nm of 0.3. Chloramphenicol, which is a bacteriostatic antibiotic (Clewell, 1972), was then added and the cells incubated for a further 90min. Cycloserine, which incorporates into the cell wall of growing cells causing lysis (Curtiss et al., 1965), was then added, and the optical density at 595nm of the culture measured at 0.5h intervals until the optical density had fallen to a stable value. The unlysed cells were harvested by

centrifugation at 12000xg for 20min at room temperature, and the pellet washed twice with 20ml LBT. The final pellet was resuspended in 5ml LBT and appropriate dilutions were plated out on rich media. After incubation for 18h at 42°C, single colonies from these plates were patched onto plates of rich medium and incubated for 18h at 42°C. Master plates were replicated onto rich media and rich media containing chloramphenicol, and chloramphenicol-sensitive isolates were purified.

2.10 DNA-DNA HYBRIDIZATION

2.10A PROBE SYNTHESIS

Probes were prepared by primed synthesis using the 350 base pair Sau3A restriction endonuclease-generated fragment of the pstC-containing plasmid pAN92 as template and a synthetic random 10-mer as primer. The reaction contained, in 20µl, 100ng of template DNA and 1µg of primer which had been heated together at 100°C for 3min and quickly chilled to 0°C, 50mM sodium phosphate, pH6.8, 10mM $MgCl_2$, 10mM DTT, 500µM dCTP, 500µM dGTP, 500µM dTTP, 3µM (variable) [α-^{32}P]dATP (approx. 111TBq/mmol; 370MBq/ml), and 2.5U of E. coli DNA polymerase I large fragment, and was allowed to proceed for 2h at 37°C. Incorporation into acid precipitable material (Maniatis et al., 1982) was 70%. The probe was used without purification by including ATP (1mM) and sodium pyrophosphate (1mM) in the prehybridization and hybridization fluid (Ullrich et al., 1984). Probe DNA was denatured before use by heating for 5min at 100°C, and quickly chilling.

2.10B HYBRIDIZATION

The procedure employed was essentially as described by Silhavy *et al.* (1984). Chromosomal or plasmid DNA was first subjected to restriction endonuclease digestion, followed by agarose gel (0.8%, w/v) electrophoresis. The gel was then stained in ethidium bromide and photographed (see Section 2.5B), and soaked for 1h at room temperature in 50mM NaOH to denature the DNA. After immersion for 30min at room temperature in distilled water, the gel was transferred to Whatman 3MM paper, covered with Saran wrap, and dehydrated thoroughly in a gel dryer (Bio-Rad Model 483 Slab Dryer). The Saran wrap was peeled away, and the gel separated from the Whatman paper by re-hydration.

The gel was then placed in a plastic container and slowly agitated for 21h at 42°C with 100ml of a solution containing:

5x SSC
5x Denhardt's solution
50mM sodium phosphate, pH6.5
50% formamide (v/v)
50µg/ml sonicated salmon sperm DNA

SSC contained 0.15M NaCl and 0.015M sodium citrate, and was made up as a 20-fold concentrate and adjusted to pH7.0 with NaOH. Denhardt's solution contained 0.02% each of Ficoll, polyvinyl pyrrolidone (M_r 360,000), and bovine serum albumin, and was made up as a 100-fold concentrate in 3x SSC.

Probe DNA, ≈4×10^6 cpm/ml (specific activity ≈8×10^8 cpm/μg DNA), was then added to the above solution containing the gel, and incubated for 14h at 42°C with slow agitation. The solution was then decanted and the gel washed thoroughly, as follows, with:

1. 2x SSC, 1x Denhardt's solution for 15min at room temperature, and repeated.

2. 2x SSC, 0.1% SDS for 30min at room temperature, and repeated three times.

3. 0.1x SSC, 0.1% SDS for 45min at 42°C with slow agitation and repeated twice.

For each wash 200ml of solution was used. The elution of background radioactivity was followed with a handheld monitor. After the final wash excess liquid was blotted from the gel with absorbent paper. The gel was placed on a sheet of used X-ray film and covered with Saran wrap. Autoradiography of the gel was performed as described in Section 2.8G.

2.11 PROTEIN MANIPULATIONS

2.11A AMINO ACID SEQUENCING

Automated amino acid sequencing was performed by Denis C. Shaw (Department of Physical Biochemistry, John Curtin School of Medical Research, Australian National University) on a Beckman

890C sequencer with a 1M Quadrol programme. Residues were identified, after manual conversion to the phenylthiohydantoin derivatives, by high-pressure liquid chromatography on a Hewlett-Packard model 1084B instrument with an Altex 165 variable wavelength detector; the column was Zorbax ODS (Du Pont Co., Wilmington, Del.) with elution by sodium acetate-acetonitrile. Any ambiguity was resolved by amino acid analysis after hydrolysis in HCl-$SnCl_2$ (Mendez and Lai, 1975).

2.11B PEPTIDE MAPPING AND AMINO ACID ANALYSIS

Peptide mapping and amino acid analysis of peptides was performed by Denis C. Shaw, while the amino acid composition of purified proteins was determined by Lewis B. James (Department of Biochemistry, John Curtin School of Medical Research, Australian National University). Peptide mapping was done as described previously (Gerdes and Rosenberg, 1974). Proteins or peptides were hydrolysed in 6M HCl at 100°C for 22h. The acid was removed by rotary evaporation and the amino acids dissolved in 0.2M sodium citrate buffer, pH2.2. Amino acid analysis was carried out in a Beckman 120B analyser as described by Spackman et al. (1958) with ninhydrin reagent prepared with titanous chloride (James, 1978); or in a Beckman System 6300 high-performance analyser.

CHAPTER 3

THE DNA SEQUENCE OF THE GENES ENCODING THE PST SYSTEM

3.1 INTRODUCTION

At the time of commencement of this study, there was considerable confusion over the number, identity and arrangement of the genes which comprised the phosphate-specific transport (Pst) system (see Chapter 1, Section 1.4C1). The only protein component of this system that had been described was the phosphate-binding protein (PBP), the product of the *phoS* gene (Gerdes and Rosenberg, 1974). The other genes comprising the Pst system, *phoT*, *pstB*, *pstA* and *phoU*, had been defined by mutations. The proteins encoded by these genes had not been identified.

It was decided to sequence the DNA encoding the Pst system, to identify open reading frames (ORFs) which could potentially encode proteins. Information derived from amino acid analysis of the purified PBP (Gerdes and Rosenberg, 1974) would identify the *phoS* gene. The other ORFs would be related to genes, as defined by mutations, through the construction, and use in genetic complementation of known mutant alleles, of plasmids carrying only one ORF (see Chapter 4). The DNA sequence would also facilitate the identification of all protein components of the Pst system (Chapter 4), and *in vitro* manipulation of cloned DNA (Chapters 5 and 6). The knowledge of gene organization thus obtained would engender an understanding of the regulation of the Pst system.

3.2 AMINO ACID ANALYSIS OF THE PHOSPHATE-BINDING PROTEIN

Phosphate-binding protein, purified from the shock-fluid of strain AB3311 as previously described (Gerdes and Rosenberg, 1974), was a gift from H. Rosenberg. The N-terminal amino acid sequence of the purified PBP was determined by automated amino acid sequencing (Chapter 2, Section 2.11A). The first twelve amino acids of the native protein were found to be:

```
1                   5                   10
Glu-Ala-Ser-Leu-Thr-Gly-Ala-Gly-Ala-Thr-Phe-Pro
```

Several chymotryptic-tryptic peptides were prepared from the purified PBP and subjected to amino acid analysis (see Chapter 2, Section 2.11B). The compositions of four chymotryptic-tryptic peptides (termed P1, P2, P3, and P4, respectively) were: Glx (Asx, Thr, Gly) Lys; (Asx, Ser, Glx, Pro, Ala, Val_2, Ile, Leu) Arg; (Glx, Val) Arg; (Ile_2, Leu, His) Lys. This information was subsequently used to determine the *phoS* reading frame (see Section 3.5).

3.3 LOCALIZATION OF GENES SPECIFYING THE PST SYSTEM

The genes which encode the phosphate-specific transport system and those that encode the polypeptide subunits of the proton-translocating ATPase (*unc*) are located less than 0.5 min apart on the chromosome of *E. coli* K12 (Bachmann, 1983). Downie *et al.* (1980) had cloned the *unc* genes on two HindIII fragments,

which are both present on plasmid pAN45 (Fig. 3.1). The genes specifying the Pst system are located on the larger HindIII restriction endonuclease-generated fragment of pAN45 (Cox et al., 1981). This fragment is also present on plasmid pAN36 (Fig. 3.1) (Cox et al., 1981). Plasmid pAN92 (Jans et al., 1983) and plasmid pAN127 (Cox et al., 1981) are two recombinant plasmids which also carry genes specifying the components of the Pst system (Fig. 3.1).

Earlier work in this laboratory showed that plasmid pAN92 complemented mutations affecting the pstA, pstB, phoT and phoS genes (G.B. Cox and H. Rosenberg, personal communication). Plasmid pAN127 also complemented mutations in the pstA, pstB and phoT genes but not mutations affecting the phoS gene (Cox et al., 1981). It was concluded that plasmid pAN92 carried the phoS gene, whereas plasmid pAN127 did not (Fig. 3.1). Furthermore, the region of DNA immediately to the left of the PstI site of plasmid pAN92 (Fig. 3.1) is part of the uncB gene (Gay and Walker, 1981). The phoS gene, therefore, must lie within the 2.3kb PstI-BstEII fragment of plasmid pAN92 and may overlap the BstEII site.

To define the location of the other genes which encode the Pst system more precisely the 1.3kb PvuI fragment was deleted from plasmid pAN127 (Fig. 3.2). This plasmid was digested with the restriction endonuclease PvuI, and re-ligated. The ligation mixture was used to transform strain AN1459 (ilvC thr leu recA). Transformants were selected for chloramphenicol resistance on

Fig. 3.1. Physical maps of plasmids pAN45, pAN92 and pAN127. Restriction endonuclease sites: A, AvaI; B, BstEII; H, HpaI; Hi, HindIII; M, MluI; P, PstI. The physical map of plasmid pAN36 is the same as that for plasmid pAN45, with the exception that the 4.4kb HindIII fragment carrying the uncB gene is absent. Plasmid pAN92 was derived from plasmid pAN45 by deletion of the 10kb PstI fragment. Plasmid pAN127 was derived from plasmid pAN36 by the introduction of a large deletion after PstI digestion and transformation with linear DNA. The broken line in the restriction map for plasmid pAN127 indicates the extent of the deletion. The approximate locations of genes on the various plasmids are as shown. Parentheses indicate that the relative order of genes had not been established. The correct order of genes is presented in Chapter 4. In the map of plasmid pAN45 only the HindIII and the PstI restriction sites are shown. The scale used to represent plasmid pAN45 (upper-left portion of the figure) is twice that for plasmids pAN92 and pAN127. The order of genes shown left to right in the plasmids corresponds to the counterclockwise direction on the E. coli genetic map. kb, kilobase pair.

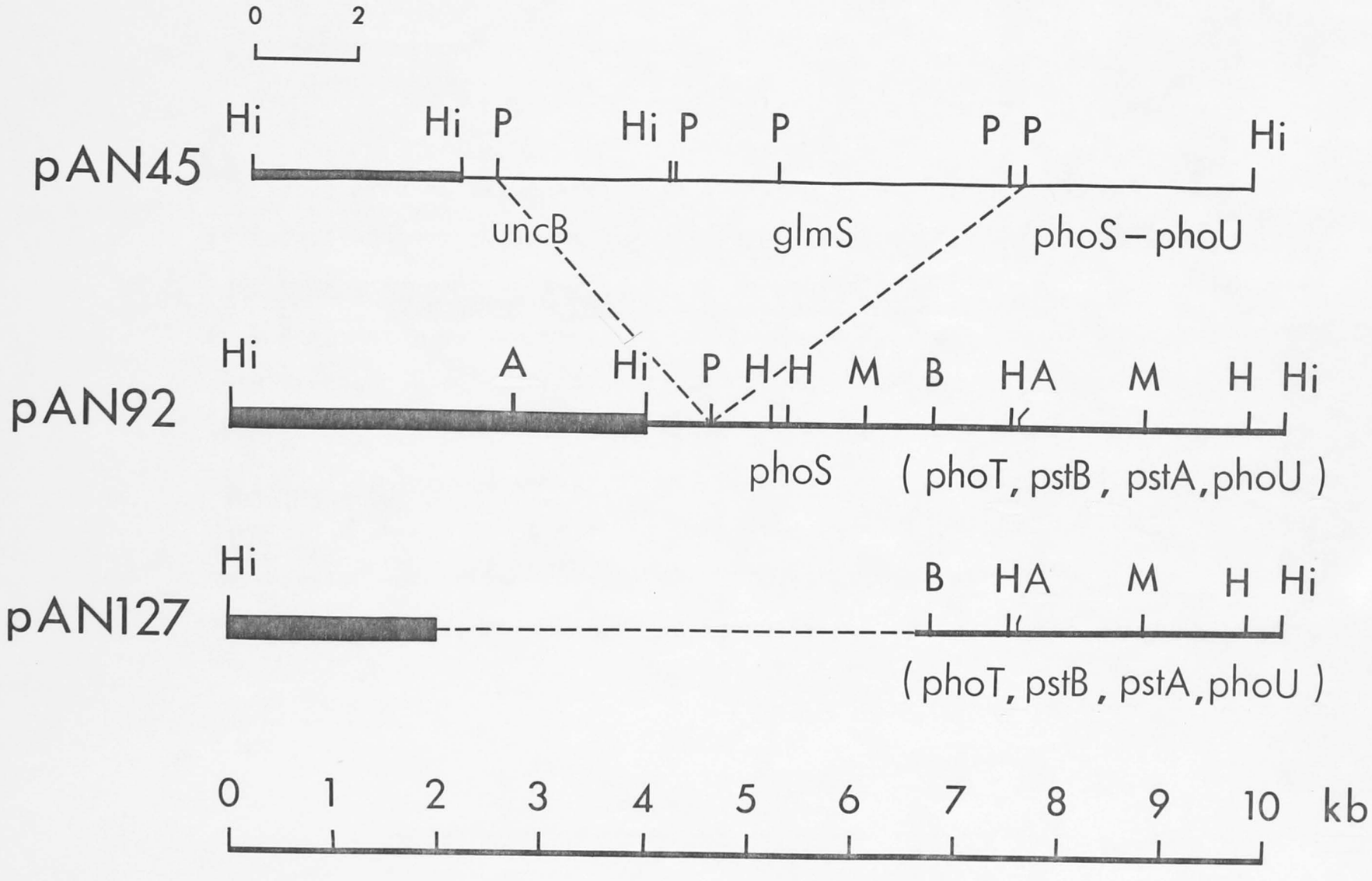
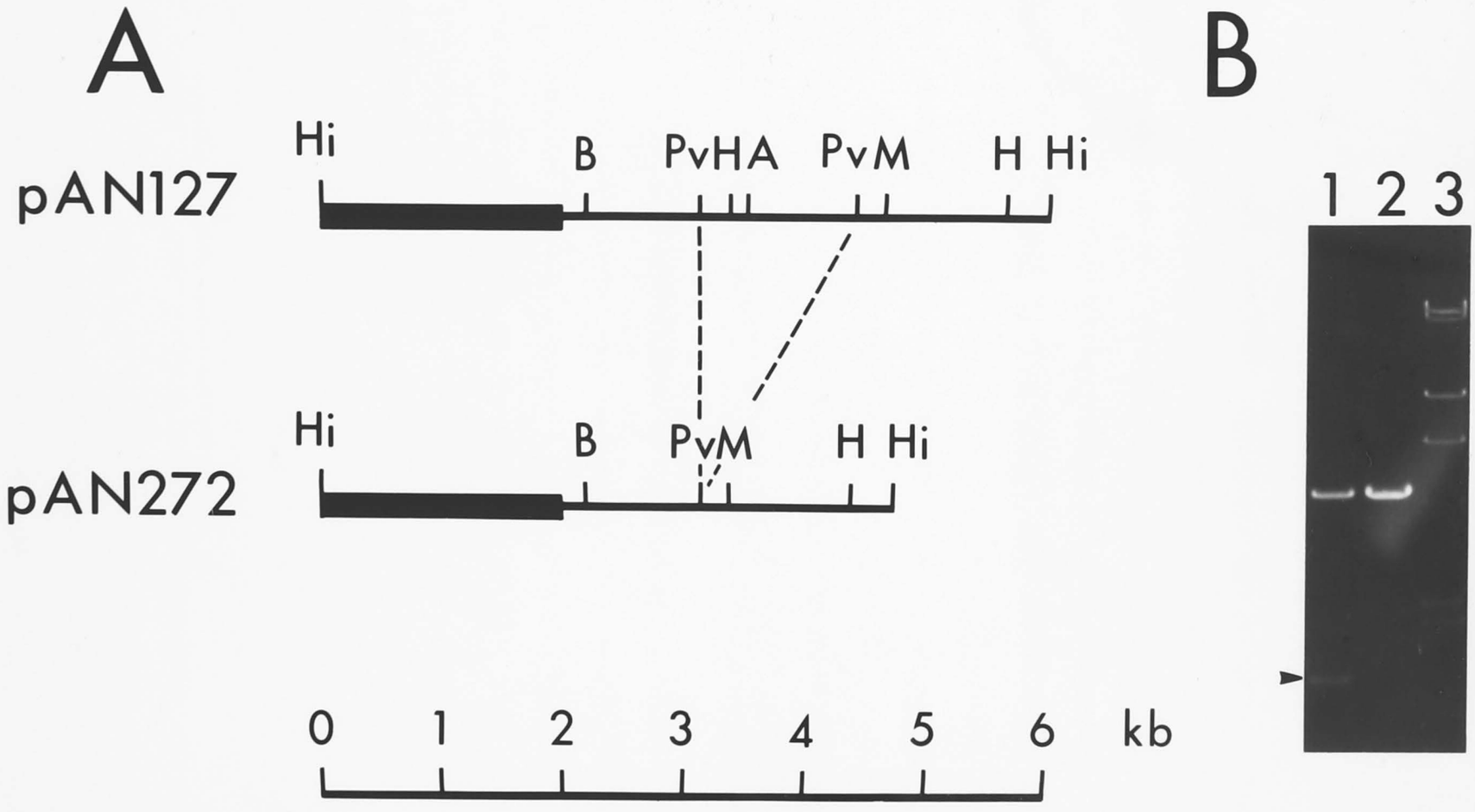

0 2
pAN45
Hi Hi P Hi P P P P Hi
uncB glmS phoS – phoU
pAN92
Hi A Hi P H H M B HA M H Hi
phoS (phoT, pstB , pstA, phoU)
pAN127
Hi B HA M H Hi
(phoT, pstB , pstA, phoU)
0 1 2 3 4 5 6 7 8 9 10 kb

Fig. 3.2. A. Restriction maps of plasmids pAN127 and pAN272. The restriction endonuclease sites are: A, AvaI; B, BstEII; H, HpaI; Hi, HindIII; M, MluI; Pv, PvuI. kb, kilobase pair. Plasmid pAN272 was derived from plasmid pAN127 by deletion of the 1.3kb PvuI fragment. B. Agarose gel electrophoresis of plasmid DNA. Plasmids pAN127 and pAN272 were digested with the restriction endonuclease PvuI and electrophoresed on a 0.6% (w/v) agarose gel, in the presence of λ DNA digested with the restriction endonuclease HindIII as molecular weight marker. The 1.3kb fragment, present in plasmid pAN127 but absent in plasmid pAN272, is indicated with an arrowhead. (1) pAN127; (2) pAN272; (3) λ DNA.


A
B
pAN127
Hi
B
PvHA
PvM
H
Hi
pAN272
Hi
B
PvM
H
Hi
0
1
2
3
4
5
6
kb
1
2
3

rich medium, and individual transformants screened for the presence and size of recombinant plasmids using the plasmid sizing procedure (Chapter 2, Section 2.6H). A typical transformant adjudged to contain a plasmid about one kb smaller than plasmid pAN127 was used as a source of plasmid DNA, which was purified by ultracentrifugation on a caesium chloride gradient of cleared lysate derived from the transformant (Chapter 2, Section 2.6A). The purified plasmid, pAN272 (Fig. 3.2A), was then digested with the restriction endonuclease PvuI, and the digest electrophoresed on an agarose gel (0.6%, w/v) together with a similar digest of plasmid pAN127 (Fig. 3.2B). It is clear that plasmid pAN272 has lost the 1.3kb PvuI fragment that is present in plasmid pAN127.

The ability of plasmid pAN272 to complement mutations in the pst region of the chromosome was then checked, using plasmid pAN127 as a control. These plasmids were used to transform each of the strains carrying the pstA2, phoT32, pstB401, and phoU35 alleles, respectively. Transformants were selected for chloramphenicol resistance on rich medium, and purified on selective media in the presence of the antibiotic. Individual transformants were grown up in the high Pi minimal medium in the presence of chloramphenicol, and a periplasmic-cytoplasmic fraction prepared from each transformant as described in Chapter 2 (Section 2.3C). The alkaline phosphatase activity was measured in each fraction (see Chapter 2, Section 2.4A), and complementation was assumed to occur when little or no activity, which is indicative of the phosphate-repressible phenotype, was detected.

Alkaline phosphatase activity was detected in all the transformants carrying plasmid pAN272, but not in the transformants carrying plasmid pAN127 (Table 3.1). It was concluded that plasmid pAN272, in contrast to plasmid pAN127, complemented neither of the four mutant alleles (*pstA2*, *phoT32*, *pstB401* and *phoU35*). The 1.3kb *Pvu*I fragment, present in plasmid pAN127, but absent from plasmid pAN272, thus carried either part, or all, of the *pstA*, *phoT*, *pstB* and *phoU* genes. This conclusion was in agreement with the results of Amemura *et al.* (1982), with the exception that these authors did not investigate the *pstB* locus.

It was decided to use plasmids pAN92 and pAN127 for sequencing the DNA carrying the *pst* genes, using the procedure of Maxam and Gilbert (1980). The following regions were sequenced: in plasmid pAN92, the 2.3kb *Pst*I-*Bst*EII fragment, and in plasmid pAN127 the 1.3kb *Pvu*I fragment, and its flanking region.

3.4 RESTRICTION MAPPING AND SEQUENCING STRATEGY

A detailed restriction map of plasmid pAN92 and plasmid pAN127 was first constructed, to formulate a strategy for DNA sequencing. The restriction endonucleases *Hin*fI and *Taq*I were mainly used, as both have four base-pair recognition sites, to yield fragments generally of a size convenient for DNA sequencing. In addition fragments generated by digestion with either restriction endonuclease can be labelled at the 3'-ends, using DNA polymerase I (Klenow fragment) and either [α-^{32}P]dATP

Table 3.1. Alkaline phosphatase activity of mutant and transformed strains.

The alkaline phosphatase activity of the periplasmic-cytoplasmic fractions of the strains carrying the mutations indicated below, and of their plasmid-bearing derivatives, was determined (Chapter 2, Section 2.4A) after growth on the high phosphate minimal medium (Chapter 2, Section 2.2D).

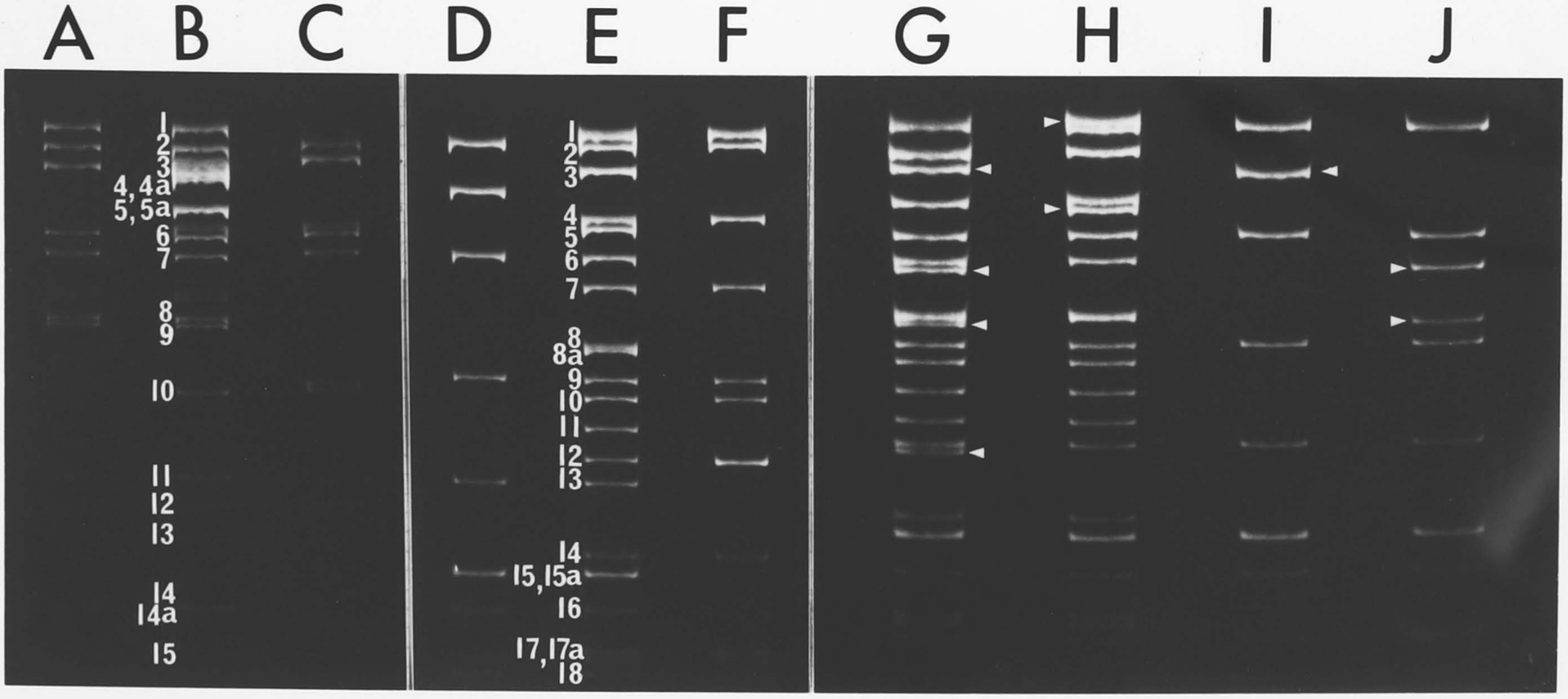
Transforming Plasmid	Mutant allele (on chromosome)			
	pstA2	phoT32	pstB401	phoU35
None	0.5[a]	1.1	2.0	1.0
pAN127	<0.1	<0.1	<0.1	<0.1
pAN272	0.5	1.2	2.1	1.2

[a] Alkaline phosphatase activities are expressed as μmol of ρ-nitrophenol formed/min per mg protein.

(for HinfI fragments) or [α-^{32}P]dCTP (for TaqI fragments).

The strategy involved identification of the HinfI and TaqI restriction endonuclease-generated fragments from plasmid pAN92 and plasmid pAN127, which originated from the chromosomal DNA insert, as opposed to those from the plasmid vector pACYC184. Plasmids pAN92, pAN127 and pACYC184 were digested with the restriction endonucleases HinfI and TaqI, and the digests were electrophoresed on an 8% w/v (30:1) acrylamide gel (see Chapter 2, Section 2.5A), and compared (Fig. 3.3). The bands present in the restriction pattern for plasmid pAN92 but absent from that for plasmid pACYC184 were derived from the chromosomal DNA insert. Plasmid pAN92 but not plasmid pAN127 complemented mutations in the phoS gene (see above). The bands derived from the insert and present in the pattern for plasmid pAN92, but absent from plasmid pAN127, must therefore represent part of the phoS gene. Since plasmid pAN92 and plasmid pAN127 complemented mutations affecting the pstA, pstB, phoT and phoU genes (see above), those bands present in the electrophoretic pattern for both plasmids pAN92 and pAN127, but absent from the pattern for plasmid pACYC184, must originate from the region of DNA carrying these genes. The bands present in the pattern for plasmid pACYC184, absent from that for plasmid pAN92, were clearly the HinfI and TaqI restriction fragments of the vector into which the HindIII chromosomal fragment carrying the genes specifying the Pst system had been inserted. Digestion of plasmids pAN92 and pACYC184 with the restriction endonucleases HinfI or TaqI, and HindIII, confirmed this (Fig. 3.3).

Fig. 3.3. Polyacrylamide gel electrophoresis of restriction endonuclease cleavage fragments of plasmid DNA. Samples were run on an 8% (w/v) gel as described in Chapter 2, Section 2.6I. (A) pAN127 digested with TaqI; (B) pAN92 digested with TaqI; (C) pACYC184 digested with TaqI; (D) pACYC184 digested with HinfI; (E) pAN92 digested with HinfI; (F) pAN127 digested with HinfI; (G) pAN92 digested with HinfI and HindIII; (H) pAN92 digested with HinfI; (I) pACYC184 digested with HinfI; (J) pACYC184 digested with HinfI and HindIII. Arrows denote the HinfI restriction endonuclease-generated bands from plasmids pAN92 and pACYC184 that are cut by HindIII in (H) and (I); and indicate the HindIII-digestion products of these bands in (G) and (J) (see Section 3.4).


A
B
C
D
E
F
G
H
I
J
1
2
3
4, 4a
5, 5a
6
7
8
9
10
11
12
13
14
14a
15
1
2
3
4
5
6
7
8
8a
9
10
11
12
13
14
15, 15a
16
17, 17a
18

To determine which HinfI and TaqI restriction endonuclease-generated fragments contained the AvaI, BstEII, HpaI, MluI, PstI and PvuI restriction sites of plasmids pAN92 and pAN127, digests were performed with each of these enzymes subsequent to HinfI or TaqI restriction. A typical gel is shown in Fig. 3.4. This approach enabled a HinfI/TaqI restriction map of the DNA fragment carrying the genes specifying the Pst system to be established (Fig. 3.5). The region of DNA to the left of the PstI restriction site of plasmid pAN92 is part of the uncB gene (Gay and Walker, 1981). Identification of HinfI and TaqI restriction endonuclease-generated fragments in this region was facilitated by comparison of experimentally determined restriction fragment sizes with the published DNA sequence for the uncB gene (Gay and Walker, 1981). The information derived from the restriction endonuclease mapping and which is summarised in Fig. 3.5 was used to formulate a strategy for DNA sequencing (Fig. 3.6). The 4.7kb PstI-PvuI fragment, and its flanking region, of plasmid pAN92 (Fig. 3.5) was sequenced using this strategy. This DNA fragment with its flanking region was sufficient for the complementation of all mutations which affected the Pst system (see Section 3.3 above).

There were regions of DNA that could not be sequenced by using end-labelled HinfI or TaqI restriction endonuclease-generated fragments. These regions were generally located more than 250-300 nucleotides from the HinfI or TaqI restriction sites, and therefore were beyond the scope of resolution of the sequencing reactions used (Maxam and Gilbert, 1980). This necessitated the use of the restriction endonucleases DpnI, Sau3A

Fig. 3.4. Polyacrylamide gel electrophoresis of restriction endonuclease cleavage fragments of plasmid DNA. Samples were run on an 8% (w/v) gel as described in Chapter 2, Section 2.6I. (A) pAN92 digested with HinfI and MluI; (B) pAN92 digested with HinfI and HpaI; (C) pAN92 digested with HinfI and BstEII; (D) pAN92 digested with HinfI and AvaI; (E) pAN92 digested with HinfI; (F) and (I) pAN127 digested with HinfI; (G) pAN127 digested with HinfI and HpaI; (H) pAN127 digested with HinfI and AvaI; (J) pAN127 digested with HinfI and BstEII. Arrows denote the position of the HinfI restriction endonuclease-generated bands from plasmids pAN92 and pAN127 that are cut by the second restriction endonuclease used. A double arrow indicates the presence of two sites for the second restriction endonuclease. DNA fragments are numbered according to the convention used in Fig. 3.3E. Fragment No. 7 carries a HpaI restriction endonuclease site that was detected only by DNA sequencing.

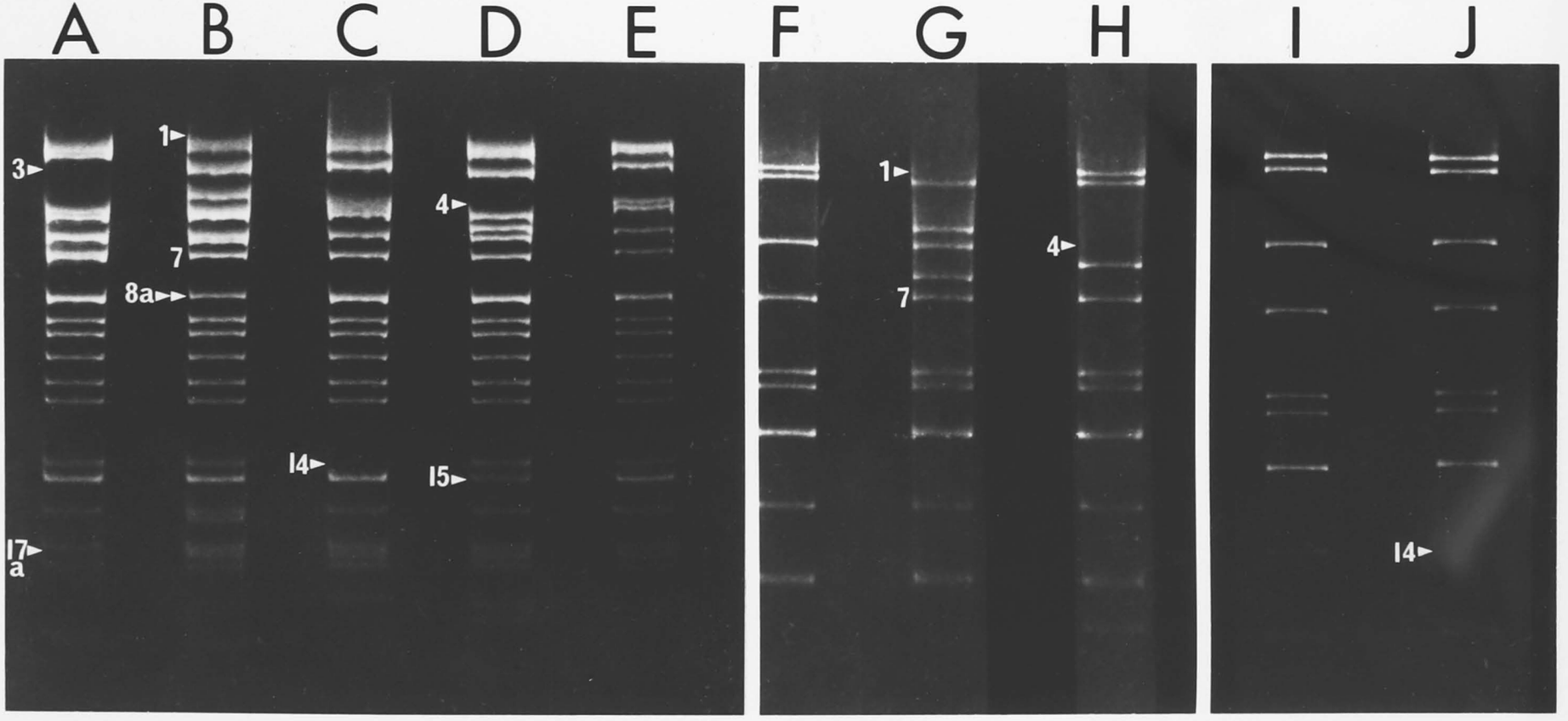

A
B
C
D
E
F
G
H
I
J
3
1
7
8a
14
4
15
17a
1
7
4
14

Fig. 3.5. Restriction endonuclease fragment map for the chromosomal DNA insert of plasmid pAN92. The HinfI and TaqI fragments of plasmid pAN92 (see Fig. 3.3), are shown in relation to one another, and to the sites for the restriction endonucleases indicated. The approximate positions of the phoS gene, ORF-1, ORF-2, ORF-3 and ORF-4, determined by DNA sequencing (see Section 3.5), are shown. The order of genes and open reading frames shown left to right in the figure corresponds to the counterclockwise direction on the E. coli genetic map. The dashed arrow indicates the extent of the chromosomal DNA insert of plasmid pAN127. The map was derived as described in Section 3.4 (see Figs. 3.3 and 3.4). Restriction endonuclease sites: A, AvaI; B, BstEII; H, HpaI; Hi, HindIII; M, MluI; P, PstI; Pv, PvuI.

pAN92

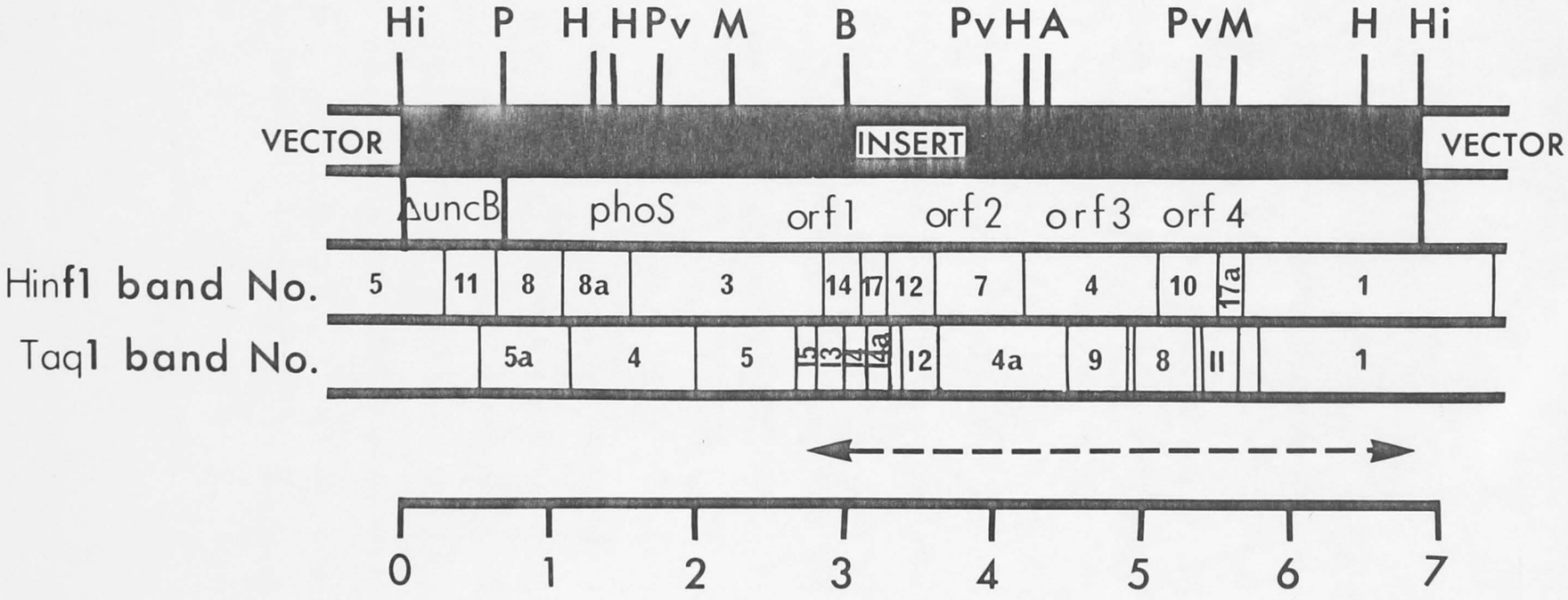
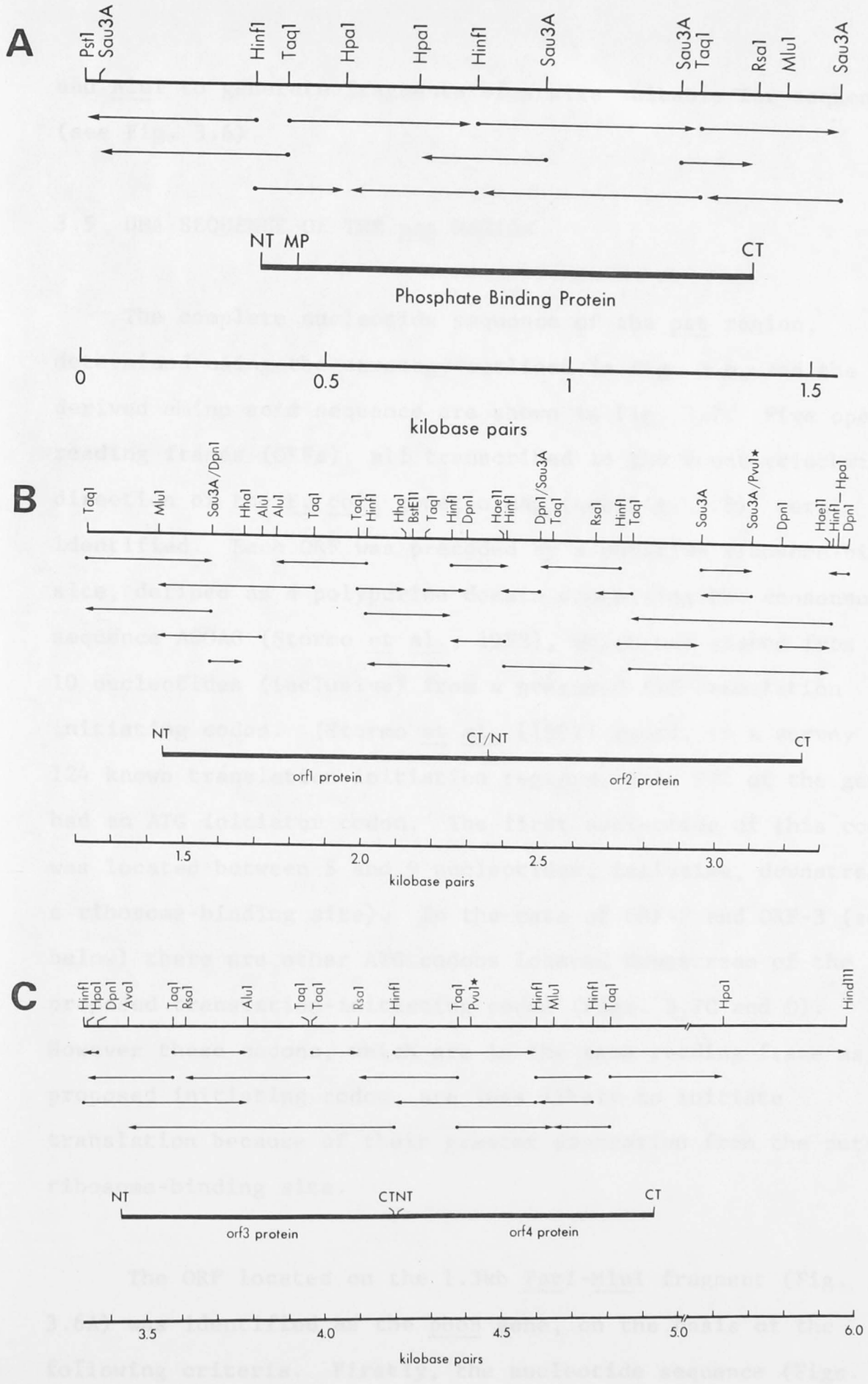

Hi
P
H
HPv
M
B
PvHA
PvM
H
Hi
VECTOR
INSERT
VECTOR
ΔuncB
phoS
orf1
orf2
orf3
orf4
Hinf1 band No.
5
11
8
8a
3
14
17
12
7
4
10
17a
1
Taq1 band No.
5a
4
5
15
13
14
14a
12
4a
9
8
11
1
0
1
2
3
4
5
6
7
kilobase pairs

Fig. 3.6. Sequencing strategy for the phoS gene (A), for ORF-1 and ORF-2 (B) and for ORF-3 and ORF-4 (C). Restriction sites for the enzymes AluI, AvaI, BstEII, DpnI, HaeII, HhaI, HindIII, HinfI, HpaI, MluI, PstI, PvuI, RsaI, Sau3A and TaqI were determined as described in Section 3.4 and were verified from the nucleotide sequence. With the exception of the MluI site (in A), the BstEII and HpaI sites (in B), and the PvuI sites (in B and C), only those restriction sites used in the sequencing experiments are shown. The PvuI sites used in the construction of plasmid pAN272 (see Fig. 3.2) are marked (*). In C an arbitrary break of 0.5kb has been introduced between the TaqI and the HpaI sites. The position of the ^{32}P label is indicated (•). The arrows show the restriction sites used to separate ^{32}P-labelled ends. Where strand separation was used to separate ^{32}P-labelled ends, an arrow links two similar restriction sites. The location of the structural genes for the phosphate-binding protein (PBP), and the ORF-1, ORF-2, ORF-3, and ORF-4 proteins, is shown with respect to the region of DNA sequenced. The location of the amino termini shown for the PBP and the ORF-4 protein have been confirmed (see text). NT, Proposed amino terminus; CT, proposed carboxyl terminus.


A
Pst1
Sau3A
Hinf1
Taq1
Hpa1
Hpa1
Hinf1
Sau3A
Sau3A
Taq1
Rsa1
Mlu1
Sau3A
NT MP
CT
Phosphate Binding Protein
0
0.5
1
1.5
kilobase pairs
B
Taq1
Mlu1
Sau3A/Dpn1
Hha1
Alu1
Alu1
Taq1
Taq1
Hinf1
Hha1
BstE11
Taq1
Hinf1
Dpn1
Hae11
Hinf1
Dpn1/Sau3A
Taq1
Rsa1
Hinf1
Taq1
Sau3A
Sau3A/Pvu1★
Dpn1
Hae11
Hinf1
Dpn1
Hpa1
NT
CT/NT
CT
orf1 protein
orf2 protein
1.5
2.0
2.5
3.0
kilobase pairs
C
Hinf1
Hpa1
Dpn1
Ava1
Taq1
Rsa1
Alu1
Taq1
Taq1
Rsa1
Hinf1
Taq1
Pvu1★
Hinf1
Mlu1
Hinf1
Taq1
Hpa1
Hind111
NT
CTNT
CT
orf3 protein
orf4 protein
3.5
4.0
4.5
5.0
6.0
kilobase pairs

and AluI to generate fragments of a size suitable for sequencing (see Fig. 3.6).

3.5 DNA SEQUENCE OF THE pst REGION

The complete nucleotide sequence of the pst region, determined using the strategy outlined in Fig. 3.6, and the derived amino acid sequence are shown in Fig. 3.7. Five open reading frames (ORFs), all transcribed in the counterclockwise direction of the E. coli genetic map (see Fig. 3.5), were identified. Each ORF was preceded by a putative ribosome-binding site, defined as a polypurine domain containing the consensus sequence AGGAG (Stormo et al., 1982), which was spaced from 6 to 10 nucleotides (inclusive) from a presumed ATG translation initiating codon. (Stormo et al. [1982] found, in a survey of 124 known translation initiation regions, that 97% of the genes had an ATG initiator codon. The first nucleotide of this codon was located between 5 and 9 nucleotides, inclusive, downstream of a ribosome-binding site). In the case of ORF-2 and ORF-3 (see below) there are other ATG codons located downstream of the proposed translation-initiating codon (Figs. 3.7C and D). However these codons, which are in the same reading frame as the proposed initiating codon, are less likely to initiate translation because of their greater separation from the putative ribosome-binding site.

The ORF located on the 1.3kb PstI-MluI fragment (Fig. 3.6A) was identified as the phoS gene, on the basis of the following criteria. Firstly, the nucleotide sequence (Figs. 3.7

Fig. 3.7. Nucleotide sequence of the pst region and the deduced amino acid sequence of the gene products. (A) The phoS gene; (B) ORF-1; (C) ORF-2; (D) ORF-3 and ORF-4. The proposed point for initiation of translation in each case is indicated by an arrow followed by the name of the gene or ORF. The sequence presented (non-transcribed strand) is numbered taking the position of the first nucleotide of the translational initiating codon (ATG) of the phoS gene as +1. Nucleotides representing part of the potential ribosome-binding site are marked by an open circle. Asterisks denote the proposed translational stop codons.

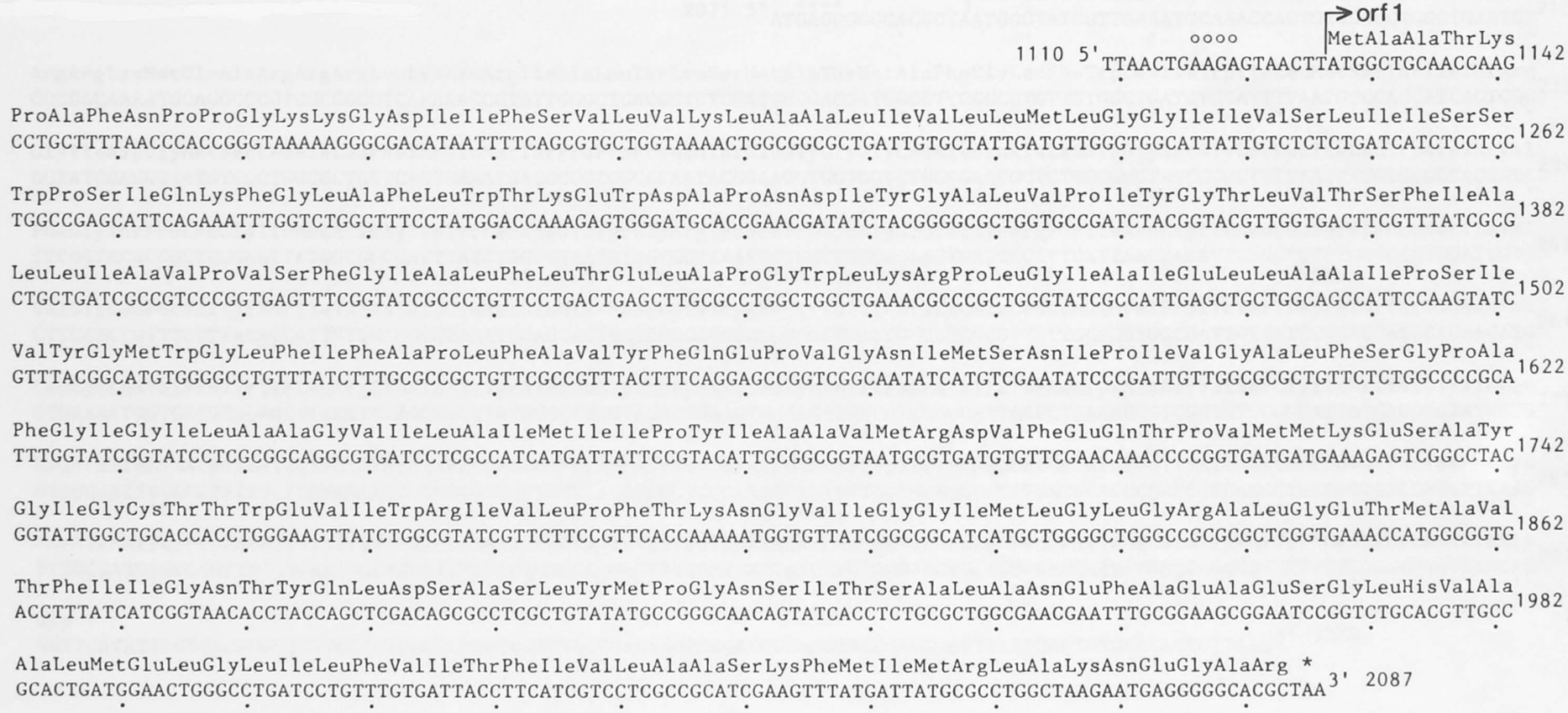
In A the nucleotide sequence of a segment of DNA located at the 3' end of the glmS gene (Walker et al., 1984) and the derived amino acid sequence, is shown upstream of the phoS gene. The amino acid sequences of the N-terminus and of four chymotryptic-tryptic peptides (P1, P2, P3 and P4) (see Section 3.2) of the phosphate-binding protein (PBP) are underlined. P1 is the closest, and P4 the furthest, peptide from the N-terminal glutamate residue of the mature PBP.

In D the experimentally verified N-terminus of the purified ORF-4 protein (see Chapter 7) is underlined.

3.7A


LeuAlaLysSerValThrValGlu * *
-339 5'
-280
CTGGCAAAATCGGTTACGGTTGAGTAATAAATGGAGCCCTGCGTAAGCGGGGCATTTTTC
TTCCTGTTATGTTTTTAATCAAACATCCTGCCAACTCCATGTGACAAAACCGTCATCTTCGGCTACTTTTTCTCTGTCACAGAATGAAAATTTTTCTGTCATCTCTTCGTTATTAATGTTT -160
GTAATTGACTGAATATCAACGCTTATTTAAATCAGACTGAAGACTTTATCTCTCTGTCATAAAACTGTCATATTCCTTACATATAACTGTCACCTGTTTGTCCTATTTTGCTTCTCGTAG -40
phoS
oooo
MetLysValMetArgThrThrValAlaThrValValAlaAlaThrLeuSerMetSerAlaPheSerValPheAlaGluAla
CCAACAAACAATGCTTTATGAATCCTCCCAGGAGACATTATGAAAGTTATGCGTACCACCGTCGCAACTGTTGTCGCCGCGACCTTATCGATGAGTGCTTTCTCTGTGTTTGCAGAAGCA 81
SerLeuThrGlyAlaGlyAlaThrPheProAlaProValTyrAlaLysTrpAlaAspThrTyrGlnLysGluThrGlyAsnLysValAsnTyrGlnGlyIleGlySerSerGlyGlyVal
AGCCTGACAGGTGCAGGTGCAACCTTCCCTGCGCCGGTGTATGCCAAATGGGCTGACACTTACCAGAAAGAAACCGGTAATAAAGTTAACTACCAGGGTATCGGTTCTTCCGGTGGCGTA 201
LysGlnIleIleAlaAsnThrValAspPheGlyAlaSerAspAlaProLeuSerAspGluLysLeuAlaGlnGluGlyLeuPheGlnPheProThrValIleGlyGlyValValLeuAla
AAACAGATTATCGCTAATACCGTTGATTTTGGTGCCTCTGACGCGCCGCTGTCTGACGAAAAACTGGCTCAGGAAGGTCTGTTCCAGTTCCCGACCGTGATTGGCGGCGTGGTGCTGGCG 321
ValAsnIleProGlyLeuLysSerGlyGluLeuValLeuAspGlyLysThrLeuGlyAspIleTyrLeuGlyLysIleLysLysTrpAspAspGluAlaIleAlaLysLeuAsnProGly
GTTAACATTCCAGGGCTGAAGTCTGGCGAACTGGTGCTGGATGGTAAAACCCTCGGCGACATCTACCTGGGCAAAATCAAGAAGTGGGATGATGAAGCCATCGCCAAACTGAATCCGGGT 441
LeuLysLeuProSerGlnAsnIleAlaValValArgArgAlaAspGlySerGlyThrSerPheValPheThrSerTyrLeuAlaLysValAsnGluGluTrpLysAsnAsnValGlyThr
CTGAAACTGCCTTCACAAAACATTGCTGTAGTACGCCGCGCAGATGGCTCCGGGACTTCCTTCGTCTTCACCAGCTACCTGGCGAAAGTGAACGAAGAGTGGAAAAACAACGTTGGTACT 561
GlySerThrValLysTrpProIleGlyLeuGlyGlyLysGlyAsnAspGlyIleAlaAlaPheValGlnArgLeuProGlyAlaIleGlyTyrValGluTyrAlaTyrAlaLysGlnAsn
GGCTCTACCGTAAAATGGCCGATCGGTCTGGGCGGTAAAGGTAACGACGGTATCGCCGCGTTCGTTCAGCGTCTGCCGGGTGCAATTGGTTATGTTGAATATGCTTACGCGAAGCAGAAC 681
AsnLeuAlaTyrThrLysLeuIleSerAlaAspGlyLysProValSerProThrGluGluAsnPheAlaAsnAlaAlaLysGlyAlaAspTrpSerLysThrPheAlaGlnAspLeuThr
AACCTGGCGTACACCAAACTGATCTCCGCTGATGGTAAACCGGTTAGTCCGACCGAAGAAAACTTCGCTAATGCAGCAAAAGGTGCAGACTGGAGCAAAACCTTCGCTCAGGATCTGACC 801
AsnGlnLysGlyGluAspAlaTrpProIleThrSerThrThrPheIleLeuIleHisLysAspGlnLysLysProGluGlnGlyThrGluValLeuLysPhePheAspTrpAlaTyrLys
AACCAGAAAGGCGAAGATGCATGGCCTATTACCTCTACCACGTTCATTCTGATCCACAAAGATCAGAAGAAACCAGAACAAGGCACAGAAGTGCTGAAATTCTTCGACTGGGCGTACAAA 921
ThrGlyAlaLysGlnAlaAsnAspLeuAspTyrAlaSerLeuProAspSerValValGluGlnValArgAlaAlaTrpLysThrAsnIleLysAspSerSerGlyLysProLeuTyr * 1041
ACCGGGGCTAAACAGGCGAACGACCTGGATTACGCCAGCCTGCCGGATAGTGTAGTTGAACAGGTTCGCGCTGCGTGGAAGACCAATATTAAAGACAGTAGCGGTAAGCCGCTGTACTAA
*
TAAAACTCCAGGCCGGGTACGGTGTTTTACGCCGCATCCGGCATTACAAAATGACTTTGTAAACGCGT 3' 1109

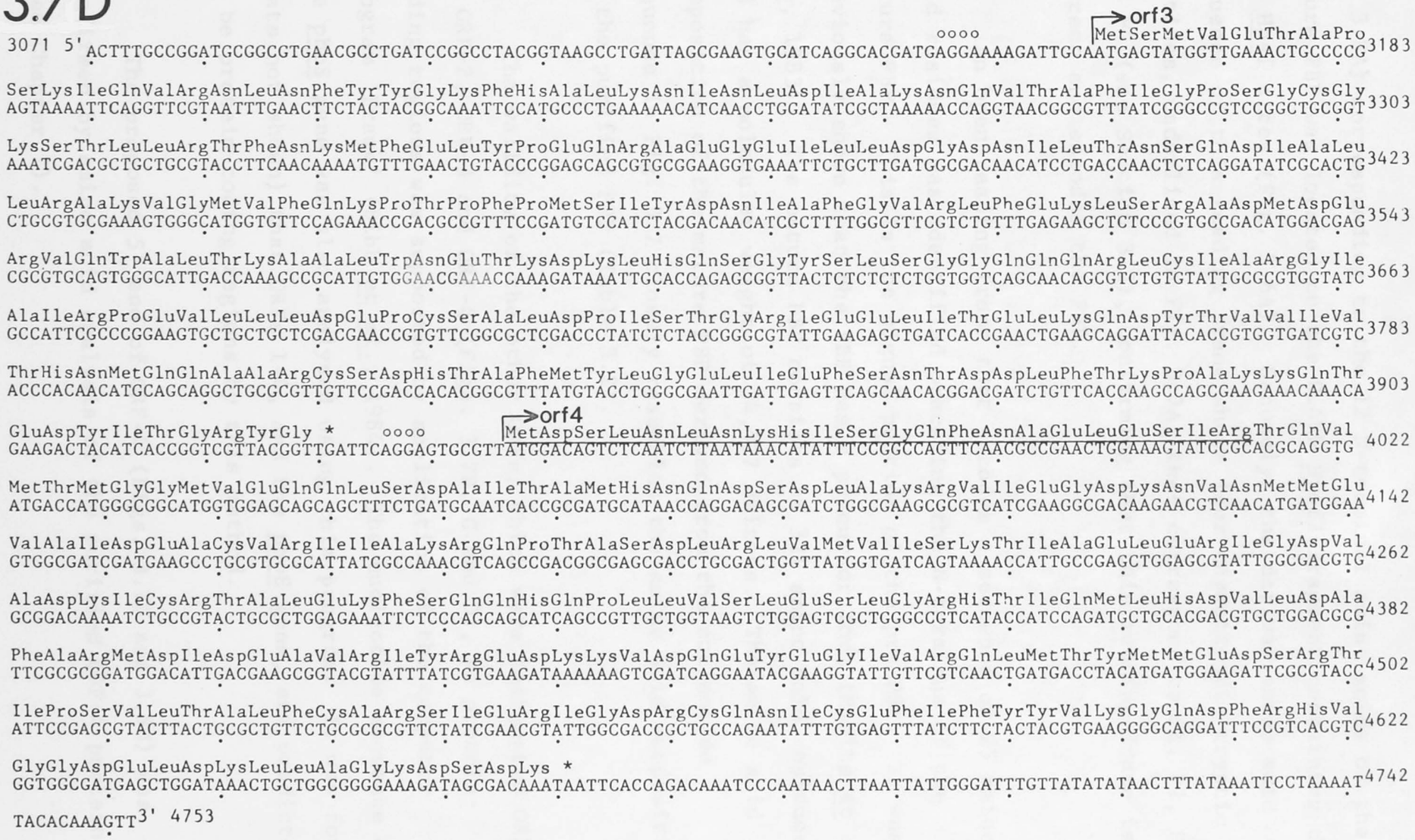
3.7B


orf 1
MetAlaAlaThrLys
1110 5' TTAACTGAAGAGTAACTTATGGCTGCAACCAAG 1142
ProAlaPheAsnProProGlyLysLysGlyAspIleIlePheSerValLeuValLysLeuAlaAlaLeuIleValLeuLeuMetLeuGlyGlyIleIleValSerLeuIleIleSerSer
CCTGCTTTTAACCCACCGGGTAAAAAGGGCGACATAATTTTCAGCGTGCTGGTAAAACTGGCGGCGCTGATTGTGCTATTGATGTTGGGTGGCATTATTGTCTCTCTGATCATCTCCTCC 1262
TrpProSerIleGlnLysPheGlyLeuAlaPheLeuTrpThrLysGluTrpAspAlaProAsnAspIleTyrGlyAlaLeuValProIleTyrGlyThrLeuValThrSerPheIleAla
TGGCCGAGCATTCAGAAATTTGGTCTGGCTTTCCTATGGACCAAAGAGTGGGATGCACCGAACGATATCTACGGGGCGCTGGTGCCGATCTACGGTACGTTGGTGACTTCGTTTATCGCG 1382
LeuLeuIleAlaValProValSerPheGlyIleAlaLeuPheLeuThrGluLeuAlaProGlyTrpLeuLysArgProLeuGlyIleAlaIleGluLeuLeuAlaAlaIleProSerIle
CTGCTGATCGCCGTCCCGGTGAGTTTCGGTATCGCCCTGTTCCTGACTGAGCTTGCGCCTGGCTGGCTGAAACGCCCGCTGGGTATCGCCATTGAGCTGCTGGCAGCCATTCCAAGTATC 1502
ValTyrGlyMetTrpGlyLeuPheIlePheAlaProLeuPheAlaValTyrPheGlnGluProValGlyAsnIleMetSerAsnIleProIleValGlyAlaLeuPheSerGlyProAla
GTTTACGGCATGTGGGGCCTGTTTATCTTTGCGCCGCTGTTCGCCGTTTACTTTCAGGAGCCGGTCGGCAATATCATGTCGAATATCCCGATTGTTGGCGCGCTGTTCTCTGGCCCCGCA 1622
PheGlyIleGlyIleLeuAlaAlaGlyValIleLeuAlaIleMetIleIleProTyrIleAlaAlaValMetArgAspValPheGluGlnThrProValMetMetLysGluSerAlaTyr
TTTGGTATCGGTATCCTCGCGGCAGGCGTGATCCTCGCCATCATGATTATTCCGTACATTGCGGCGGTAATGCGTGATGTGTTCGAACAAACCCCGGTGATGATGAAAGAGTCGGCCTAC 1742
GlyIleGlyCysThrThrTrpGluValIleTrpArgIleValLeuProPheThrLysAsnGlyValIleGlyGlyIleMetLeuGlyLeuGlyArgAlaLeuGlyGluThrMetAlaVal
GGTATTGGCTGCACCACCTGGGAAGTTATCTGGCGTATCGTTCTTCCGTTCACCAAAAATGGTGTTATCGGCGGCATCATGCTGGGGCTGGGCCGCGCGCTCGGTGAAACCATGGCGGTG 1862
ThrPheIleIleGlyAsnThrTyrGlnLeuAspSerAlaSerLeuTyrMetProGlyAsnSerIleThrSerAlaLeuAlaAsnGluPheAlaGluAlaGluSerGlyLeuHisValAla
ACCTTTATCATCGGTAACACCTACCAGCTCGACAGCGCCTCGCTGTATATGCCGGGCAACAGTATCACCTCTGCGCTGGCGAACGAATTTGCGGAAGCGGAATCCGGTCTGCACGTTGCC 1982
AlaLeuMetGluLeuGlyLeuIleLeuPheValIleThrPheIleValLeuAlaAlaSerLysPheMetIleMetArgLeuAlaLysAsnGluGlyAlaArg *
GCACTGATGGAACTGGGCCTGATCCTGTTTGTGATTACCTTCATCGTCCTCGCCGCATCGAAGTTTATGATTATGCGCCTGGCTAAGAATGAGGGGGCACGCTAA 3' 2087

3.7C


orf2
MetAlaMetValGluMetGlnThrThrAlaAlaLeuAlaGluSer
2071 5' ATGAGGGGGCACGCTAATGGCTATGGTTGAAATGCAAACCACTGCGGCGCTGGCTGAATCT 2131
ArgArgLysMetGlnAlaArgArgArgLeuLysAsnArgIleAlaLeuThrLeuSerMetAlaThrMetAlaPheGlyLeuPheTrpLeuIleTrpIleLeuMetSerThrIleThrArg
CGCCGCAAAATGCAGGCGCGTCGCCGCCTCAAAAACCGTATTGCGCTGACGCTCTCGATGGCGACGATGGCCTTCGGCCTGTTCTGGCTGATCTGGATTTTAATGTCCACCATCACTCGC 2251
GlyIleAspGlyMetSerLeuAlaLeuPheThrGluMetThrProProProAsnThrGluGlyGlyGlyLeuAlaAsnAlaLeuAlaGlySerGlyLeuLeuIleLeuTrpAlaThrVal
GGTATCGACGGTATGTCGCTGGCGCTGTTCACTGAAATGACGCCGCCGCCCAATACGGAAGGTGGTGGTCTGGCGAACGCTCTGGCGGGTAGCGGGCTGTTAATTTTGTGGGCCACGGTA 2371
PheGlyThrProLeuGlyIleMetAlaGlyIleTyrLeuAlaGluTyrGlyArgLysSerTrpLeuAlaGluValIleArgPheIleAsnAspIleLeuLeuSerAlaProSerIleVal
TTCGGTACGCCGCTGGGCATTATGGCGGGGATTTATCTGGCGGAATATGGTCGTAAATCCTGGCTGGCAGAAGTGATTCGCTTCATTAACGACATTCTGCTCTCTGCGCCGTCGATTGTG 2491
ValGlyLeuPheValTyrThrIleValValAlaGlnMetGluHisPheSerGlyTrpAlaGlyValIleAlaLeuAlaLeuLeuGlnValProIleValIleArgThrThrGluAsnMet
GTTGGTCTGTTTGTTTACACCATTGTGGTGGCGCAGATGGAGCACTTCTCCGGCTGGGCGGGCGTGATTGCCCTGGCGTTGTTGCAGGTGCCGATTGTTATCCGCACCACCGAGAACATG 2611
LeuLysLeuValProTyrSerLeuArgGluAlaAlaTyrAlaLeuGlyThrProLysTrpLysMetIleSerAlaIleThrLeuLysAlaSerValSerGlyIleMetThrGlyIleLeu
CTGAAACTGGTGCCGTACAGCCTGCGTGAAGCGGCTTATGCGCTGGGTACACCGAAGTGGAAGATGATCTCTGCGATTACGCTGAAAGCGTCGGTGTCCGGGATTATGACCGGTATCCTG 2731
LeuAlaIleAlaArgIleAlaGlyGluThrAlaProLeuLeuPheThrAlaLeuSerAsnGlnPheTrpSerThrAspMetMetGlnProIleAlaAsnLeuProValThrIlePheLys
CTGGCGATTGCCCGTATTGCTGGTGAAACCGCGCCGCTGCTGTTTACCGCGCTCTCCAACCAGTTCTGGAGCACGGACATGATGCAGCCGATCGCCAACCTGCCGGTGACGATCTTTAAG 2851
PheAlaMetSerProPheAlaGluTrpGlnGlnLeuAlaTrpAlaGlyValLeuIleIleThrLeuCysValLeuLeuLeuAsnIleLeuAlaArgValValPheAlaLysAsnLysHis
TTTGCGATGAGCCCGTTTGCGGAATGGCAGCAATTGGCCTGGGCCGGGGTATTGATCATTACCCTGTGCGTACTGCTGCTGAACATTCTGGCGCGCGTTGTTTTTGCGAAGAATAAACAC 2971
Gly *
GGTTGATATTGCTGACACGGTTTTCCCCTCACCCTAACCCTCTCCCCAGAGGGGCGAGGGGACCGACCGAGCGCCTTTTTGACTCTGTACACGGTTAAC 3' 3070

3.7D



and 3.8A) corresponding to the 12 N-terminal amino acids of the mature PBP was located on the 0.4kb HinfI fragment containing the two HpaI sites (Fig. 3.6A). Secondly, the derived amino acid sequence of this reading frame shows four chymotryptic-tryptic peptides, underlined in Fig. 3.7A, that correspond to P1, P2, P3, and P4 (see Section 3.2), confirming that this reading frame is correct and encodes the PBP.

An open-reading frame that encodes a sequence of 25 amino acid residues was identified preceding the N-terminus of the mature PBP. This is the signal peptide of the pre-PBP. It was previously shown that the PBP has a precursor form (Morita et al., 1983). The mature PBP consists of 321 amino acid residues, and has a molecular weight of 34,427 daltons. The amino acid composition of the mature PBP derived from the nucleotide sequence in Fig. 3.7 closely resembles the amino acid composition of the purified PBP (Table 3.2).

The validity of the other ORFs, which were designated ORF-1, ORF-2, ORF-3 and ORF-4 (Figs. 3.7B, C and D), as protein-coding regions was supported by application of the computer program 'Frame' (Bibb et al., 1984). The nucleotide sequence of the phoS gene was also analysed using this program. It was found (data not shown) that ORFs 1-4, and the phoS gene, are predicted to be protein-coding regions by this method.

The proposed 5' end of ORF-4 (Figs. 3.7D and 3.8B) was confirmed by amino acid analysis of the purified ORF-4 protein (see Chapter 7).

Fig. 3.8. Sections of DNA sequencing gels from plasmids pAN92 and pAN127. A. Section from a 6% (w/v) polyacrylamide sequencing gel of a fragment, generated by the restriction endonuclease HinfI, from plasmid pAN92. The fragment (No. 8a - see Fig. 3.5) was end-filled with [α-^{32}P]dATP as the radioactive moiety, and the labelled-ends separated by digestion with the restriction endonuclease HpaI. B. Section from a 20% (w/v) polyacrylamide sequencing gel of a fragment, generated by the restriction endonuclease HinfI, from plasmid pAN127. The fragment (No. 10 - see Fig. 3.5) was end-filled with [α-^{32}P]dATP, and uniquely labelled-ends obtained by strand separation.

In A the first nucleotide of the triplet GAA which encodes the N-terminal glutamate residue of the mature PBP (see Fig. 3.7A) is marked (•). In B the arrowhead indicates the first nucleotide of the triplet ATG which specifies the N-terminal methionine residue of the ORF-4 protein, and the filled squares represent the nucleotides comprising the putative ribosome-binding site of ORF-4 (see Fig. 3.7D)

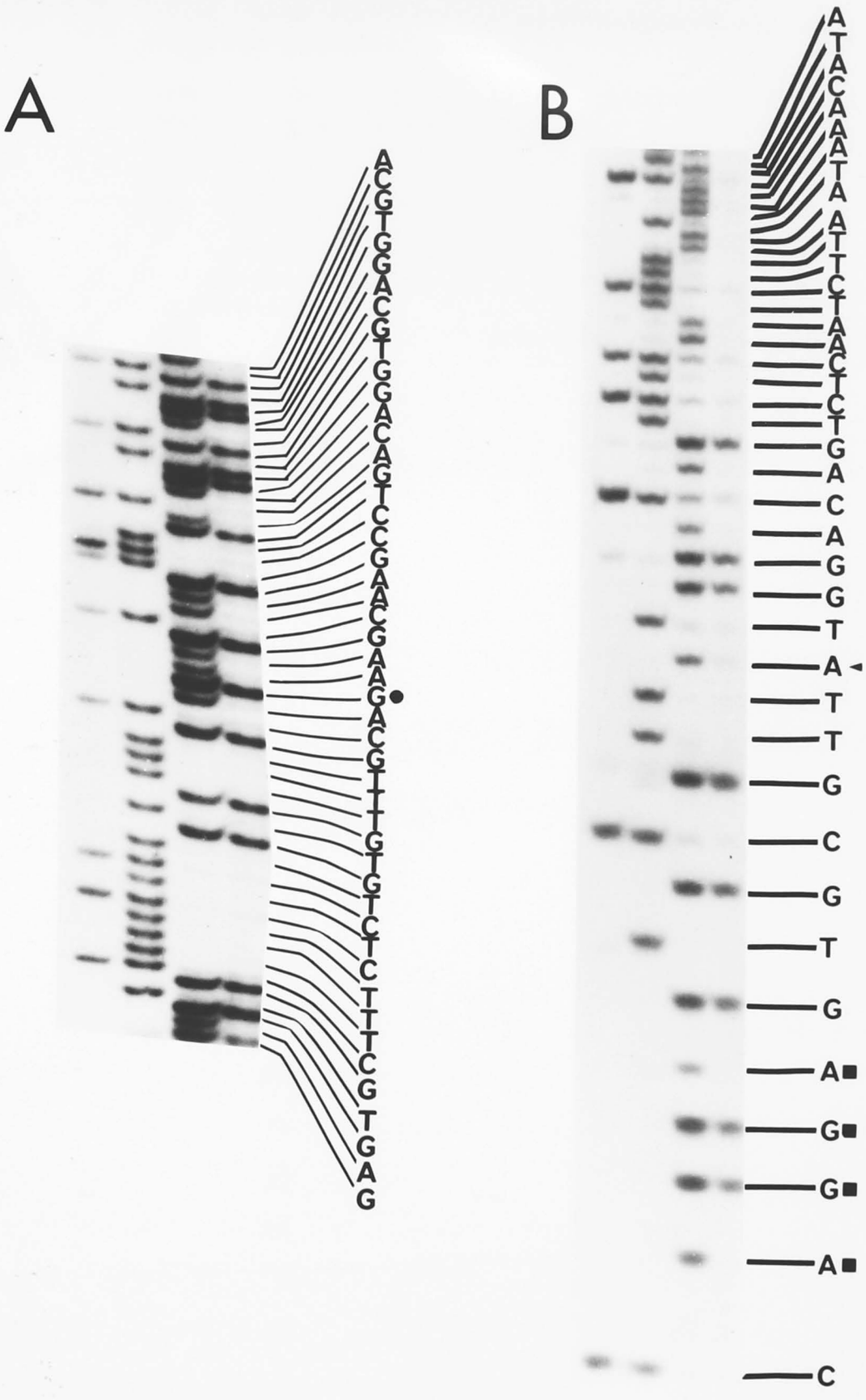

A
ACGTGGACGTGGACAGTCCGAACGAAGACGTTGTCTCTTTCGTGAG
B
ATACAAATA ATTCTAACTCTGACAGGGTA
T
T
G
C
G
T
G
A
G
G
A
C

Table 3.2. Amino acid composition of the mature phosphate-binding protein.

The amino acid composition of the purified phosphate-binding protein was determined as described in Chapter 2 (Section 2.11B).

Amino acid	Number of residues	
	Deduced from DNA sequence	Amino acid analysis of purified phosphate-binding protein
Asn	17	37
Asp	20	
Thr	21	19.8
Ser	19	18.5
Gln	14	32.4
Glu	15	
Pro	15	15.6
Gly	34	33
Ala	35	34.4
Cys	0	0
Val	22	19.7
Met	0	0
Ile	17	13.9
Leu	24	23.9
Tyr	12	11.6
Phe	12	12.1
His	1	1.7
Lys	31	29.5
Arg	4	5.3
Trp	8	ND[a]

[a]ND, Not determined.

3.6 DISCUSSION

The phoS gene and four open reading frames, designated ORF-1, ORF-2, ORF-3 and ORF-4 (see Fig. 3.7), have been identified by DNA sequencing of a cloned segment of E. coli chromosomal DNA that can complement all known mutations affecting the Pst system. The DNA sequence presented in Fig. 3.7 is in complete agreement with that reported by Magota et al. (1984) and Amemura et al. (1985). The phoS gene and ORFs 1-4 are all transcribed in the counterclockwise direction of the E. coli genetic map, opposite to that initially proposed by Cox et al. (1981).

It was confirmed that the phoS gene encodes the PBP. The N-terminal amino acid sequence and overall amino acid composition of purified PBP was in agreement with that predicted from the nucleotide sequence of the phoS gene. As seen from the amino acid composition of the PBP (Table 3.2), it has no met residues and has a high lys content, consistent with other periplasmic binding proteins (Lever, 1972). The 25 additional amino acids present in the pre-PBP constitute a typical signal peptide (Michaelis and Beckwith, 1982) with a positively charged N-terminus followed by a chain of 20 hydrophobic amino acid residues. The postulated N-terminus of the pre-PBP has been confirmed by amino acid analysis of the purified protein (Magota et al., 1984). The site of cleavage of the signal peptide to form the mature PBP lies on the carboxyl side of the alanine residue preceding the N-terminal glutamate of the mature PBP. The presence of a signal peptide in the pre-PBP is consistent with a periplasmic location for the mature PBP (Medveczky and

Rosenberg, 1970).

One structural element that has been an important feature in many models of protein secretion is a hairpin formed between the leader sequence and the mature protein (Wickner, 1979; Engelman and Steitz, 1981). The secondary and tertiary structure of the pre-PBP was predicted according to Chou and Fasman (1978). In agreement with the proposed models (result not shown) a helical hairpin structure can be predicted for the first 65 amino acids of the pre-PBP. A role for the protonmotive force in the secretion of proteins across the cytoplasmic membrane has been demonstrated (Daniels et al., 1981). Copeland et al. (1984) noted a predominance of negative charges in the first 108 amino acids of the leucine-binding proteins, and suggested that the trans-positive membrane potential may electrophorese domains of protein secondary structure that contain net negative charge across the membrane. However, the corresponding part of the PBP does not contain an excess of negatively charged amino acid residues. Thus the proposal of Copeland et al. (1984) is probably not applicable as a general mechanism for protein export. Furthermore, it has been reported (Chen and Tai, 1985) that ATP, and not the protonmotive force, is essential for posttranslational secretion of proteins into everted E. coli membrane vesicles.

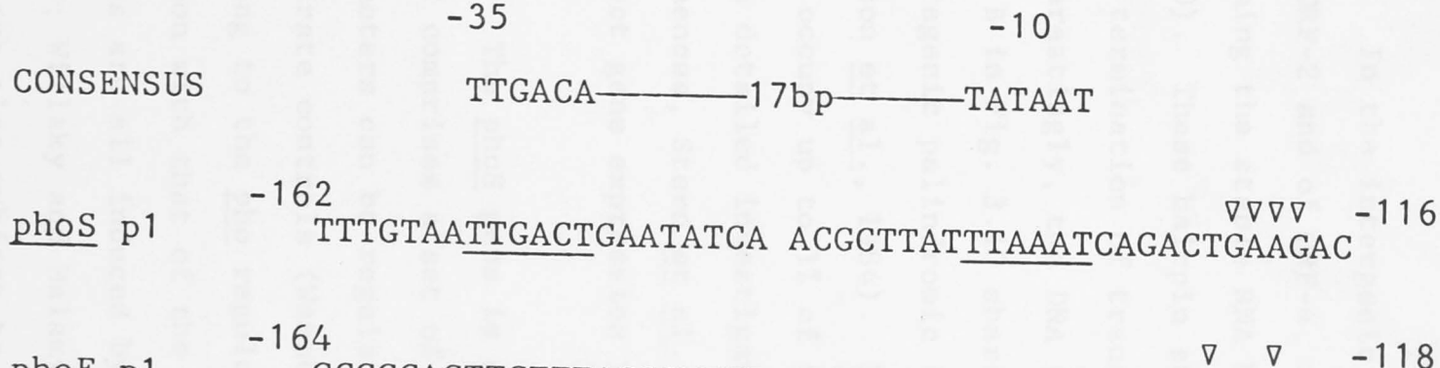
The proteins encoded by ORFs 1-4 do not possess an N-terminal peptide that has features in common with signal peptides. The ORF-1 and ORF-2 proteins have several stretches of hydrophobic amino acid residues (see Fig. 3.7) that could

presumably span the cytoplasmic membrane and thus confer on these proteins a membrane localization (Engelman and Steitz, 1981). In contrast, the proteins encoded by ORF-3 and ORF-4 do not possess such stretches of hydrophobic amino acid residues, and are probably either cytoplasmic or peripheral membrane proteins.

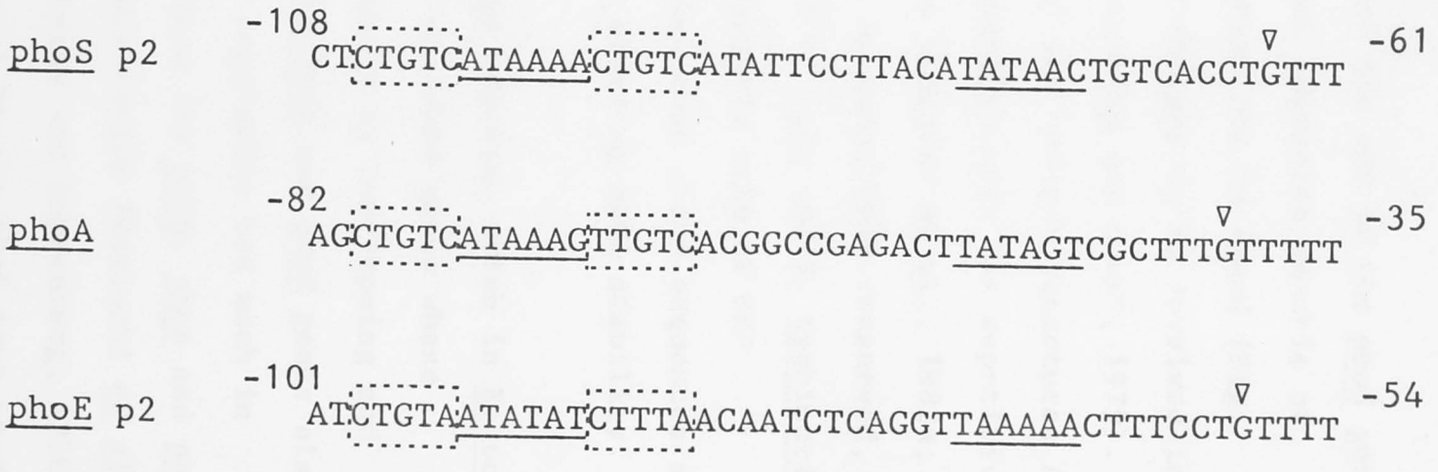
The DNA sequence preceding the start of the phoS gene, and of each open reading frame, was examined for regions that could be involved in the interaction with RNA polymerase and which are known to be highly conserved in the promoters of E. coli genes (Rosenberg and Court, 1979; Hawley and McClure, 1983). Procaryotic promoters consist of a -35 region, involved in the recognition and binding of the σ factor of RNA polymerase holoenzyme, and a -10 region (or "Pribnow box"), which constitutes the binding site for the RNA polymerase core enzyme (Pribnow, 1979). In the segments of DNA outside protein-coding sequences two regions, preceding the putative ribosome-binding site of the phoS gene, have been identified as likely functional promoters (see Fig. 3.9). One promoter region (phoS p1), consisting of the nucleotides TTAAAT (-10 region) and TTGACT (-35 region), shows very good homology with the consensus sequence for procaryotic promoters (Rosenberg and Court, 1979; Hawley and McClure, 1983). The second putative promoter (phoS p2) has a -35 region which has poor homology with the consensus promoter sequence and a -10 region which shows good homology with the consensus promoter sequence. The pentanucleotide sequence CTGTC is located on either side of the -35 region of phoS p2 (see Fig. 3.9). phoS p2 has good homology with the putative promoter of the phoA gene, the structural gene for alkaline phosphatase. In

Fig. 3.9. Comparison of the nucleotide sequences of the potential promoter regions of phoS, phoA, and phoE. (a) Nucleotide sequences of the potential promoters phoS p1 and phoE p1 (the promoter furthest from the translational start-site). (b) Nucleotide sequences of the phoS p2, phoA and phoE p2 potential promoters. The nucleotide sequences of the potential promoter regions of phoA and phoE are here numbered in the same manner as phoS (see Fig. 3.7A). This numbering differs from that used in the original publications (Kikuchi et al., 1981; Overbeeke et al., 1983). Nucleotide sequences showing homology to the consensus -35 and -10 regions are underlined. The pentanucleotide sequences on either side of the -35 region of phoS p2 and the corresponding nucleotides in the phoA p and phoE p2 are boxed. Possible start points of transcription are indicated by (▽). The nucleotide sequences of the phoS and phoE p1 potential promoters have been interrupted at an arbitrary point to produce the consensus 17 base pair (bp) interval between the -10 and -35 regions.

(a)


-35
-10
CONSENSUS
TTGACA————17bp————TATAAT
-162
phoS p1
TTTGTAATTGACTGAATATCA ACGCTTATTTAAATCAGACTGAAGAC
∇∇∇∇
-116
-164
phoE p1
GCGGCAGTTGTTTACGCTTTT ATTACAGATTTAATAAATTACCACAT
∇ ∇
-118

(b)


-108
phoS p2
CTCTGTCATAAAACTGTCATATTCCTTACATATAACTGTCACCTGTTT
∇
-61
-82
phoA
AGCTGTCATAAAGTTGTCACGGCCGAGACTTATAGTCGCTTTGTTTTT
∇
-35
-101
phoE p2
ATCTGTAATATATCTTTAACAATCTCAGGTTAAAAACTTTCCTGTTTT
∇
-54

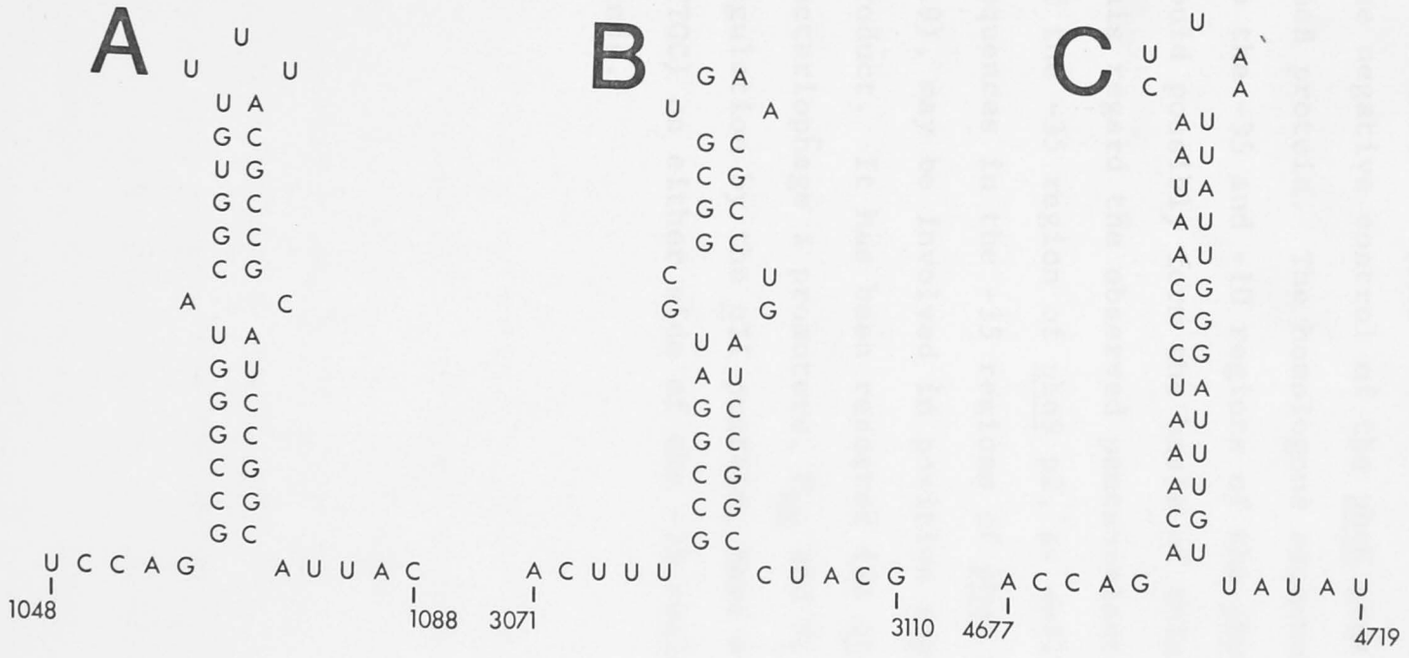
the region preceding the phoE gene (Overbeeke et al., 1983), two promoters can tentatively be identified, and these have been designated phoE p1 and phoE p2, respectively (see Fig. 3.9).

In the intergenic regions beyond the end of the phoS gene, of ORF-2 and of ORF-4, inverted repeat sequences capable of forming the stable RNA hairpin structure can be found (Fig. 3.10). These hairpin structures are thought to be involved in the termination of transcription (Rosenberg and Court, 1979). Interestingly, the DNA that comprises the hairpin structures A and B in Fig. 3.10 shares sequence homology with the repetitive extragenic palindromic (REP) sequence (Higgins et al., 1982a; Gilson et al., 1984). This sequence is remarkably conserved, and may occupy up to 1% of the genomes of E. coli and S. typhimurium. In a detailed investigation of the possible role of REP sequences, Stern et al. (1984) proposed that these sequences may affect gene expression indirectly by altering mRNA stability.

The phoS gene is part of the pho regulon, which in E. coli K-12 comprises a set of more than 20 unlinked genes whose promoters can be regulated simultaneously by overlapping and separate controls (Wanner, 1983). The phoA and phoE genes also belong to the pho regulon, and their regulation has much in common with that of the phoS gene. Thus the phoS, phoA and phoE genes are all induced by phosphate deprivation (Horiuchi et al., 1959; Willsky and Malamy, 1976; Overbeeke and Lugtenberg, 1980), and are also subject to positive control by the phoB gene product and to negative control by the phoR gene product (Morris et al., 1974; Willsky and Malamy, 1976; Tommassen and Lugtenberg, 1980).

Fig. 3.10. A, B and C: Possible mRNA secondary structures in the intergenic sequences (non-transcribed strand) of the pst region. D: Comparison of the sequences comprising Loops A and B with the consensus repetitive extragenic palindromic (REP) sequence. Numbers of the corresponding nucleotides in the DNA sequence (see Fig. 3.7) are indicated. Loop A ($\Delta G < -18.6$kcal) represents the region between the phoS gene and ORF-1. Loop B ($\Delta G = -28.4$kcal) is found between ORF-2 and ORF-3, and Loop C ($\Delta G = 21.8$kcal) depicts the region immediately beyond ORF-4. The free energy of formation (shown above in brackets for each loop) was calculated according to Tinoco et al. (1973). In D the sequence of Loop A is presented for the transcribed strand, and in the reverse orientation, to facilitate the comparison of REP sequences. The REP sequence in Loop A is in the opposite orientation to both the consensus sequence (Stern et al., 1984) and the REP sequence of Loop B.

(→) and (←) indicates transcribed and non-transcribed strand (equivalent to mRNA sequence), respectively. The full circles indicate nucleotides that are not homologous to the consensus sequence.



D

CONSENSUS G C C G/U G A U G C G G/A C G C/U G A C G C/U C U U A U C C/A G G C C U A C

phoS-orf1 ← 1083 G C C G G A U G C G G C G U A A A A C A C C G U A C C C G G C C U G G 1049

orf2-orf3 → 3076 G C C G G A U G C G G C G U G A A C G C C U G A U C C G G C C U A C 3109

In the present model of gene regulation in the pho regulon (Tommassen and Lugtenberg, 1982; Chapter 8) it is proposed that the PhoB protein is directly involved in the regulation of the phoA, phoE and phoS genes: the induction by Pi deprivation and the negative control of the phoR gene product is mediated by the PhoB protein. The homologous sequences present outside but close to the -35 and -10 regions of the phoS, phoA, and phoE genes could possibly form the basis of this regulation by phoB. In this regard the observed pentanucleotide sequences on either side of the -35 region of phoS p2, as well as the corresponding sequences in the -35 regions of phoA p and phoE p2 (see Fig. 3.9), may be involved in positive regulation by the phoB gene product. It has been reported (Ho et al., 1983) that the two bacteriophage λ promoters, P_{RE} and P_I, subject to positive regulation by the cII protein, have a tetranucleotide sequence (TTGC) on either side of the -35 regions to which the cII protein binds.

CHAPTER 4

IDENTIFICATION OF OPEN READING FRAMES WITH MUTANT ALLELES AND ASSIGNMENT OF GENE PRODUCTS

4.1 INTRODUCTION

The presence of multiple protein components in shock-sensitive transport systems has been well documented. The histidine permease (Higgins *et al.*, 1982b) and the *mgl*-dependent galactose transport system (Muller *et al.*, 1985) of *S. typhimurium*, and the maltose transport system of *E. coli* (see Hengge and Boos, 1983) have, in addition to a substrate-binding protein located in the periplasm, three proteins located in the inner membrane. The latter system differs in having a specific component, the LamB protein, which is located in the outer membrane.

It had previously been shown that mutations which resulted in the constitutive synthesis of alkaline phosphatase and in the loss of high-affinity Pi transport affected genetic loci, represented by the *phoS*, *phoT*, *pstA* and *pstB* alleles, which mapped at 83 min on the *E. coli* chromosome (Willsky *et al.*, 1973; Cox *et al.*, 1981). One of the *phoT* mutants (*phoT35*) was shown by Zuckier and Torriani (1981) to differ from the others in that it was capable of utilizing Pi in a *pit* background, and was designated *phoU* (Amemura *et al.*, 1982).

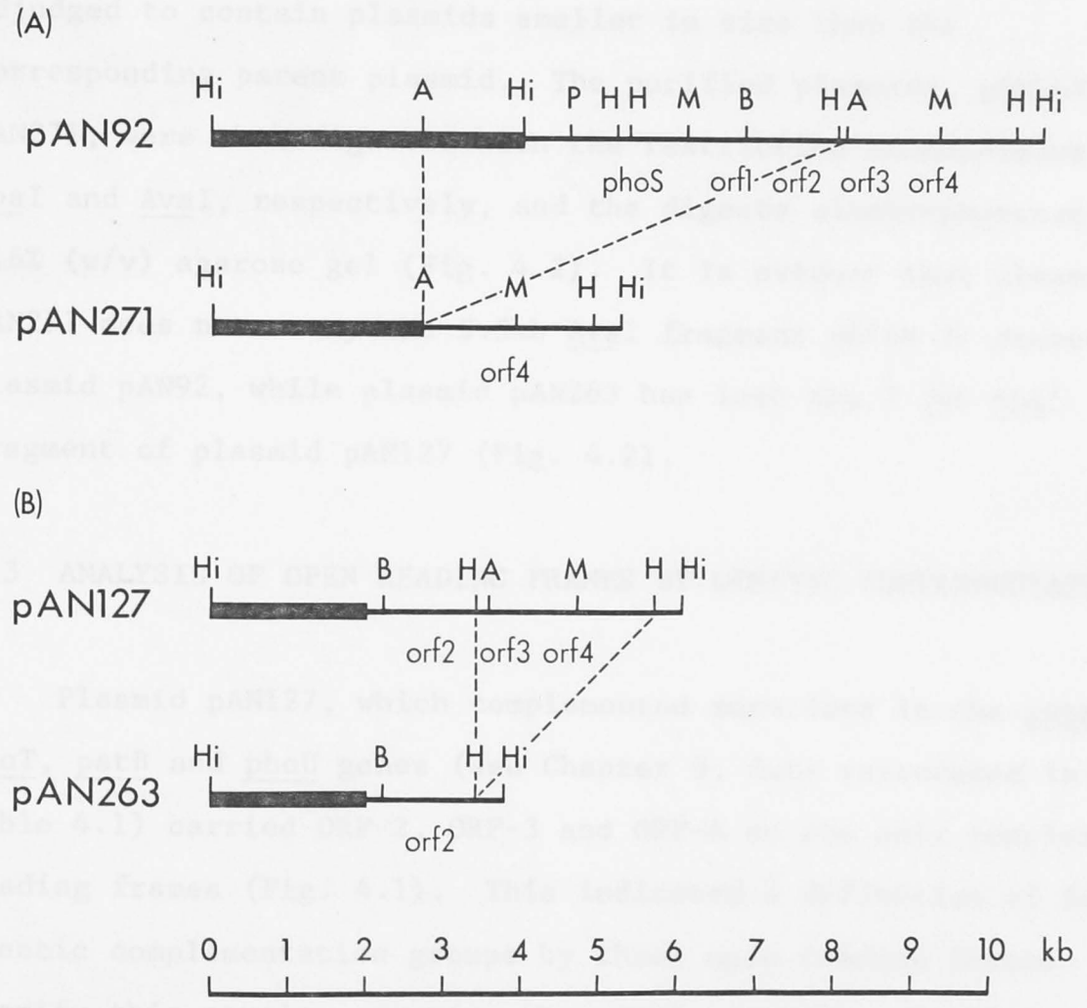
The *phoS* gene and four open reading frames (ORFs),

designated ORF-1, ORF-2, ORF-3 and ORF-4, were identified by DNA sequencing of a cloned segment of chromosomal DNA which complemented all mutations mapping in the pst region (see Chapter 3). The work to be described in this chapter concerns the relation of ORFs 1-4 with mutant alleles known to affect the Pst system, and which have been mapped in the 83 min region of the chromosome. These include the pstA2, phoT32, pstB401 and phoU35 alleles. Strains carrying these alleles were transformed with plasmids containing one ORF only, and complementation of the mutation tested. The proteins encoded by ORFs 1-4 were identified in vitro using a coupled transcription/translation system with plasmid DNA as template. The identification in vivo of the proteins comprising the Pst system was facilitated by the use of a strain carrying the multicopy plasmid pAN92, which carries the entire set of pst genes and signals necessary for their transcription (see Chapter 3).

4.2 THE CONSTRUCTION OF PLASMIDS pAN263 AND pAN271

The relation of ORFs 1-4 with known mutant alleles was facilitated by the construction of plasmids pAN263 and pAN271, which carried ORF-2 and ORF-4, respectively, as the only complete reading frame (Fig. 4.1). Plasmid pAN127 and plasmid pAN92 were digested with the restriction endonucleases HpaI and AvaI, respectively. The digested plasmids were then ligated, and the ligation mixtures separately transformed into strain AN1459 (ilvC thr leu recA). Transformants were selected for resistance to chloramphenicol on rich medium, and individual transformants were then screened for the presence and size of

Fig. 4.1. Physical maps of plasmids pAN92, pAN127, pAN263, and pAN271. Plasmid pAN271 (A) was derived from plasmid pAN92 by deletion of the 5.5kb AvaI fragment, whereas plasmid pAN263 (B) was derived from plasmid pAN127 by deleting the 2.3kb HpaI fragment. The approximate locations of the phoS gene and ORF-1, ORF-2, ORF-3, and ORF-4 (identified by DNA sequencing) on the plasmids are shown, where appropriate. Promotion of the phoS gene and ORFs 1-4 in plasmid pAN92 is regulated by the level of extracellular Pi. In the other plasmids the phoS gene and ORFs 1-4 are not Pi-regulated, and are promoted presumably from the vector DNA (see Section 4.5). The order of genes and open reading frames shown left to right in the plasmids corresponds to the counterclockwise direction of the E. coli genetic map. Restriction endonuclease sites: A, AvaI; B, BstEII; H, HpaI; Hi, HindIII; M, MluI; P, PstI. kb, kilobase pair.

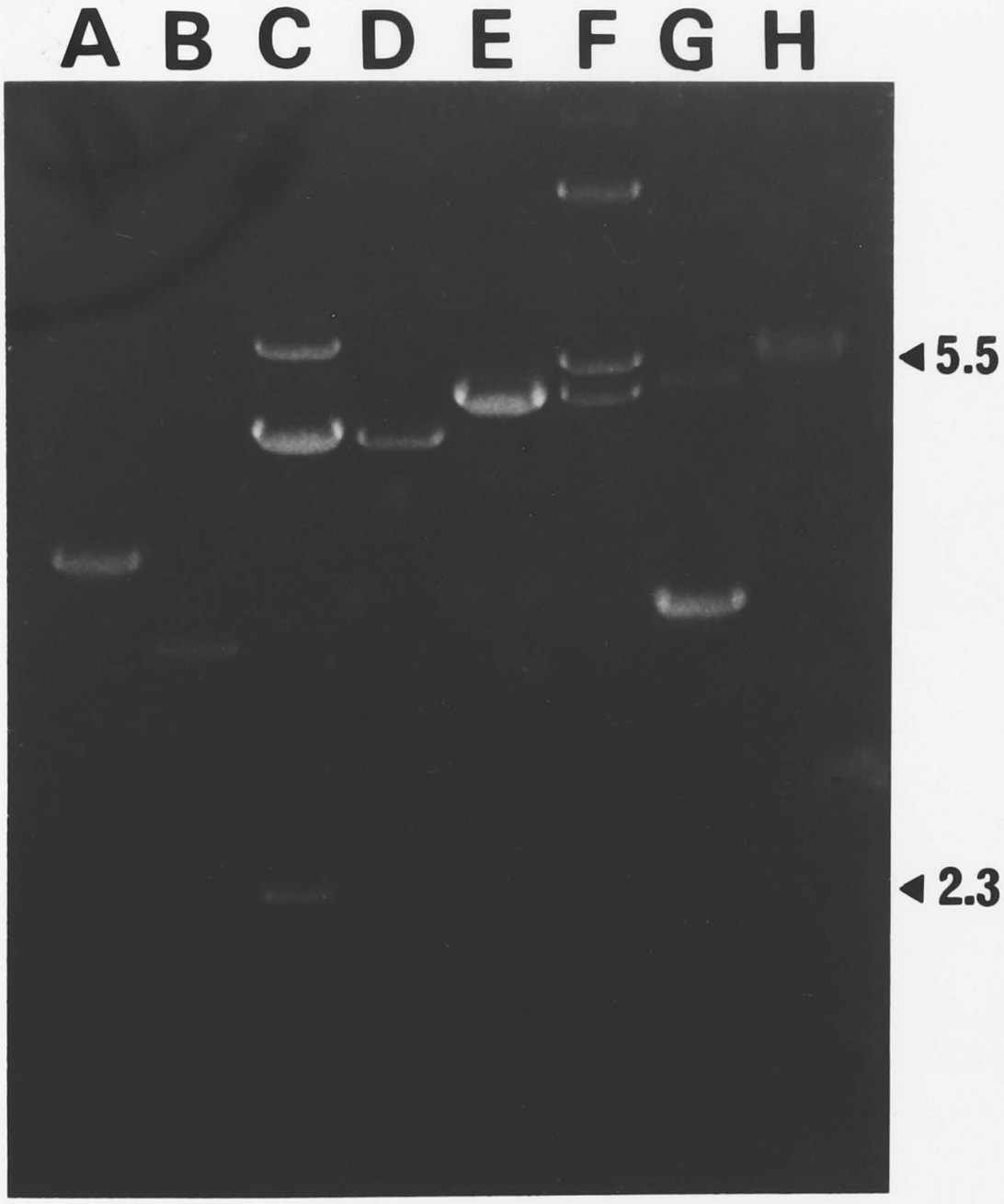

(A)
pAN92
Hi
A
Hi
P
H
H
M
B
H
A
M
H
Hi
phoS
orf1
orf2
orf3
orf4
pAN271
Hi
A
M
H
Hi
orf4
(B)
pAN127
Hi
B
H
A
M
H
Hi
orf2
orf3
orf4
pAN263
Hi
B
H
Hi
orf2
0
1
2
3
4
5
6
7
8
9
10
kb

recombinant plasmids by the plasmid sizing procedure (Chapter 2, Section 2.6H). Plasmid DNA was subsequently purified, by the ultracentrifugation of cleared lysate on a caesium chloride gradient (Chapter 2, Section 2.6A), from typical transformants adjudged to contain plasmids smaller in size than the corresponding parent plasmid. The purified plasmids, pAN263 and pAN271, were then digested with the restriction endonucleases HpaI and AvaI, respectively, and the digests electrophoresed on a 0.6% (w/v) agarose gel (Fig. 4.2). It is evident that plasmid pAN271 does not carry the 5.5kb AvaI fragment which is present in plasmid pAN92, while plasmid pAN263 has lost the 2.3kb HpaI fragment of plasmid pAN127 (Fig. 4.2).

4.3 ANALYSIS OF OPEN READING FRAMES BY GENETIC COMPLEMENTATION

Plasmid pAN127, which complemented mutations in the pstA, phoT, pstB and phoU genes (see Chapter 3, data reproduced in Table 4.1) carried ORF-2, ORF-3 and ORF-4 as the only complete reading frames (Fig. 4.1). This indicated a definition of four genetic complementation groups by three open reading frames. To clarify this strains carrying the pstA2, pstB401, phoU35 or phoT32 allele were transformed with the plasmid pAN263, which carries ORF-2 as the only complete reading frame. Transformants were selected on rich medium containing chloramphenicol, and chloramphenicol-resistant transformants were purified on selective media in the presence of the antibiotic. A plasmid-bearing derivative of each mutant was then tested for the presence of high-affinity Pi transport (Chapter 2, Section 2.4B), and for constitutive alkaline phosphatase. In the latter case

Fig. 4.2. Agarose gel electrophoresis of plasmid DNA. Plasmid DNA was digested with the restriction endonuclease HpaI or AvaI and electrophoresed on an agarose gel (0.8%, w/v), together with plasmid DNA that had not been treated with restriction endonuclease. (A) pAN127; (B) pAN263; (C) pAN127 digested with HpaI; (D) pAN263 digested with HpaI; (E) pAN271 digested with AvaI; (F) pAN92 digested with AvaI; (G) pAN271; (H) pAN92. The 2.3kb HpaI fragment of plasmid pAN127, and the 5.5kb AvaI fragment of plasmid pAN92, are indicated. Fragment sizes refer to linearized plasmid DNA.


A B C D E F G H
5.5
2.3

plasmid-bearing strains were grown in the high Pi minimal medium (Chapter 2, Section 2.2D) in the presence of chloramphenicol, and a periplasmic-cytoplasmic fraction prepared from each strain (Chapter 2, Section 2.3C). These fractions were then assayed for alkaline phosphatase activity (Chapter 2, Section 2.4A). Complementation in the transformed strains was assumed to occur with the restoration of both high-affinity Pi transport (where appropriate, see below) and the Pi-repressible alkaline phosphatase phenotype.

A functional Pst system was found in strains transformed with plasmid pAN263 and which carried either the pstA2 or phoT32 allele on the chromosome, but not in the transformant carrying the pstB401 allele on the chromosome (Table 4.1). The presence of the phoU35 allele did not affect Pi transport by the Pst system (Table 4.1). Accordingly transformants carrying the pstA2 or phoT32 allele lacked constitutive alkaline phosphatase activity, which was present in the transformants carrying the pstB401 or phoU35 allele (Table 4.1). It is concluded that the mutations pstA2 and phoT32 occur in the same gene and that this gene corresponds to ORF-2. Other phoT alleles (phoT9, phoT34) were tested and also found to be complemented by plasmid pAN263 (result not shown). It is clear that the pstA and the phoT mutations are allelic forms of the same gene, and this gene will now be referred to as pstA.

Plasmid pAN271 (Fig. 4.1), which carried ORF-4 as the only complete reading frame, was transformed into strains carrying the pstA2, pstB401, or phoU35 allele. Complementation in the

Table 4.1. Alkaline phosphatase activity and phosphate uptake rate of mutant and transformed strains.

Transforming Plasmid	Mutant allele (on chromosome)							
	pstA2		phoT32		pstB401[a]		phoU35	
	APase activity[b]	Pi uptake[c]	APase activity	Pi uptake	APase activity	Pi uptake	APase activity	Pi uptake
None	0.5	1.9	1.1	1.9	2.0	0.9	1.0	39.8
pAN127	<0.1	ND[d]	<0.1	ND	<0.1	4.4	<0.1	ND
pAN263	<0.1	26.3	<0.1	27.7	2.2	0.6	1.1	ND
pAN271	0.6	ND	1.3	ND	2.3	ND	<0.1	ND

.../contd. ...

Table 4.1. (/contd.)

[a] Cells used for the assay of phosphate uptake were grown with DL-lactate as the carbon source. An exception was strain AN1403 (*pstB401 unc-449*) which required glucose for growth. Glucose-grown cells normally show lower phosphate uptake rates than do cells grown on lactate.

[b] Alkaline phosphatase (APase) activities refer to cells grown on the high phosphate minimal medium (Chapter 2, Section 2.2D) and were measured as described in Chapter 2, Section 2.4A). The activities are expressed as μmol of ρ-nitrophenol formed/min per mg protein.

[c] Phosphate uptakes were measured as described in Chapter 2 (Section 2.4B) and are expressed as nmol of Pi/min per mg dry wt.

[d] ND, Not determined.

resulting transformants was tested by determining the alkaline phosphatase activity in the periplasmic-cytoplasmic fraction of these strains (see above). As judged by this criterion, plasmid pAN271 complemented only the *phoU35* allele (Table 4.1), thus identifying ORF-4 as the *phoU* gene.

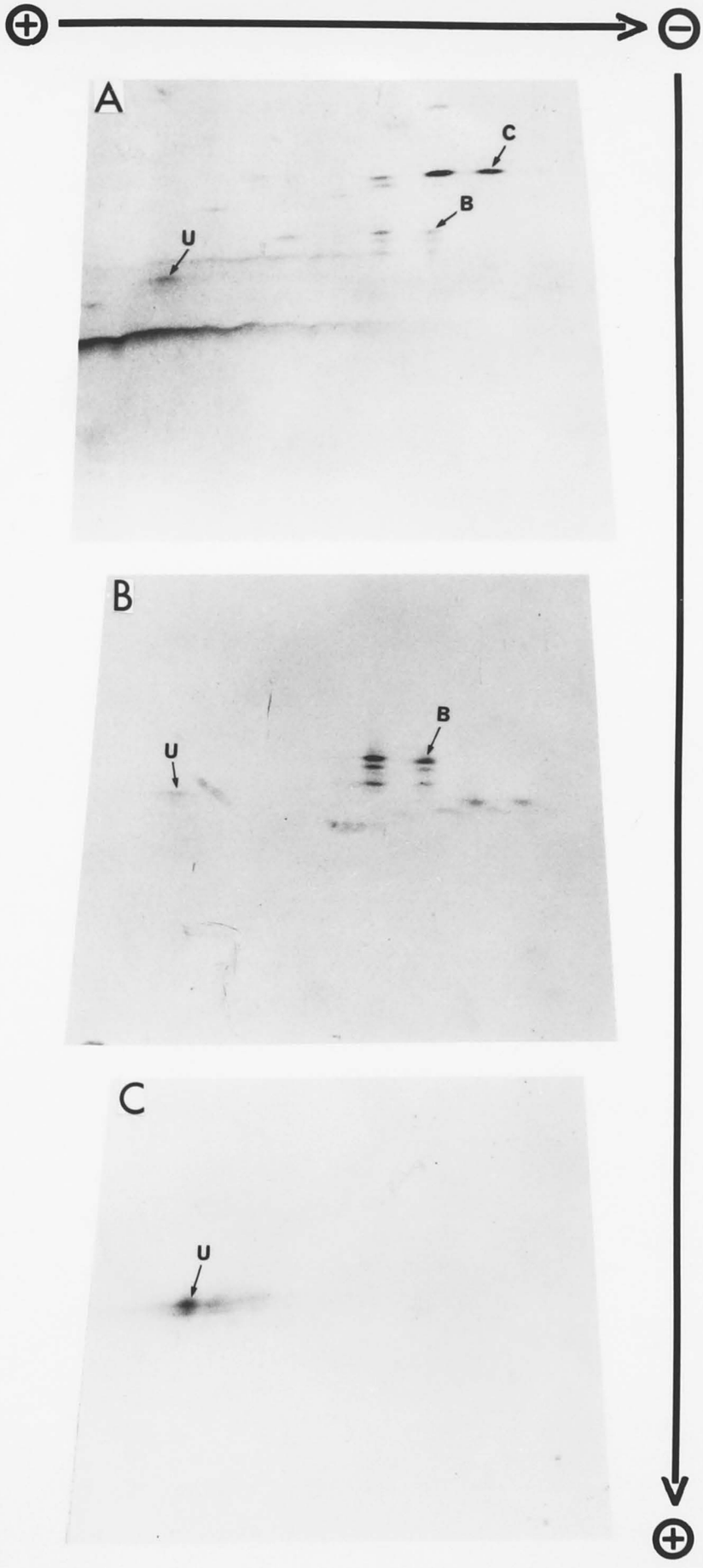
Plasmid pAN127 carried *pstA*, ORF-3, and *phoU*, but not ORF-1 (see above); as determined by the criteria outlined above, plasmid pAN127 complements the *pstB401* allele (Table 4.1). It was concluded that ORF-3 is identical with the *pstB* gene.

ORF-1 could not be related to any mutant alleles, present in our laboratory, that were known to affect the Pst system (data not shown). However it seemed likely from its putative translational coupling with the *pstA* gene (see Chapter 3, Fig. 3.7) that ORF-1 specified an essential component of the Pst system. ORF-1 was therefore given the mnemonic *pstC*.

4.4. GENE-POLYPEPTIDE RELATIONSHIPS

The *phoU* gene product was investigated using [^{35}S]methionine in an *in vitro* transcription/translation system (Chapter 2, Section 2.7) with covalently closed plasmid DNA as the template. The translation products were separated by two-dimensional gel electrophoresis (Chapter 2, Section 2.5C) and the radioactive polypeptides visualized by autoradiography of the dried gel. The *phoU* gene product was formed when plasmid pAN92 (*phoS*$^+$*pstC*$^+$*A*$^+$*B*$^+$*phoU*$^+$), pAN127 (*pstA*$^+$*B*$^+$*phoU*$^+$) or plasmid pAN271 (*phoU*$^+$) was used as DNA template (Fig. 4.3), but not from plasmid

Fig. 4.3. Autoradiographs of two-dimensional gel electrophoretograms of [^{35}S]methionine-labelled polypeptides with plasmid DNA as the template in an *in vitro* transcription/translation system. Samples of the reaction mixtures were mixed with an equal volume of lysis buffer (Chapter 2, Section 2.5C), and 100-200µl used for electrophoresis. Membranes (about 200µg of protein) prepared from strain AN1403 (*pstB401*), grown on the high Pi minimal medium (Chapter 2, Section 2.2D), and solubilized in lysis buffer were also added to each sample. The membranes had been prepared and washed in low-ionic strength buffer as described in Chapter 2 (Section 2.3C). Electrophoresis in the first dimension (isoelectric focusing) is in the horizontal direction, and in the second dimension (SDS-PAGE) in the vertical direction, of the figure. The plasmid templates were: (A) pAN92 (*phoS$^+$pstC$^+$A$^+$B$^+$phoU$^+$*); (B) pAN127 (*pstA$^+$B$^+$phoU$^+$*); (C) pAN271 (*phoU$^+$*). The PstA protein and proteins encoded by the vector plasmid pACYC184 could not be visualized under the experimental conditions used. The phosphate-binding protein was not visualized with plasmid pAN92 as DNA template because it does not contain methionine residues (Table 3.1) (Gerdes and Rosenberg, 1974) and therefore cannot be labelled with [^{35}S]methionine.


⊕
⊖
A
C
B
U
B
U
C
U
⊕

pAN263 (*pstA*$^+$) (result not shown). The single ^{35}S-labelled polypeptide in the autoradiograph of the two-dimensional gel for plasmid pAN271 is not a protein synthesized by the vector (pACYC184) portion of this plasmid. Proteins synthesized from the vector DNA could not be visualized under the conditions employed for two-dimensional gel electrophoresis, but could be seen on one-dimensional SDS-gel electrophoretograms (see Fig. 4.6 below).

To identify the PhoU protein in subcellular fractions, strain AN1667, carrying the multicopy plasmid pAN92 (see Fig. 4.1A), was grown in the low Pi minimal medium (Chapter 2, Section 2.2E). The membrane and periplasmic-cytoplasmic fractions were prepared from this strain as described in Chapter 2 (Section 2.3C) and subjected to two-dimensional gel electrophoresis. The *phoU* gene product could not be identified in the periplasmic-cytoplasmic fraction (result not shown) but was identified in the membrane fraction after coelectrophoresis with the radioactive product from the transcription/translation system (Fig. 4.4A). The PhoU protein had a molecular weight of about 26,000 daltons and an isoelectric point of about 5.4 and could be removed from the membrane by washing in low-ionic strength buffer (Fig. 4.5A). This protein was not seen in the membrane fraction from strain AN1667 grown in the high Pi minimal medium (Fig. 4.4B).

The *pstA* gene product was also identified by labelling with [^{35}S]methionine in the *in vitro* transcription/translation system using as the DNA template plasmid pAN263 (*pstA*$^+$). The product corresponding to the *pstA* gene could not be identified on two-

Fig. 4.4. Two-dimensional gel electrophoresis of membrane preparations. Samples (about 1mg) of protein from membranes which had been prepared as described in Chapter 2 (Section 2.3C) were subjected to two-dimensional gel electrophoresis (Chapter 2, Section 2.5C). Electrophoresis in the first dimension (isoelectric focusing) is in the horizontal direction, and in the second dimension (SDS-PAGE) in the vertical direction, of the figure. The labelled arrows refer to particular proteins identified by electrophoresis with authentic samples or with [^{35}S]methionine-labelled peptides, derived from *in vitro* transcription/translation. Unlabelled arrows indicate the normal positions of the absent proteins. A. membranes from strain AN1667 (pAN92/*phoS*$^+$*pstC*$^+$*A*$^+$*B*$^+$*phoU*$^+$) grown under conditions of low Pi concentration (Chapter 2, Section 2.2E). B. membranes from strain AN1667 grown under conditions of high Pi concentration (Chapter 2, Section 2.2D). C. membranes from strain AN2221 (*phoU35*) grown under conditions of high Pi concentration. D. membranes from strain AN1403 (*pstB401*) grown under conditions of high Pi concentration. Strain AN1403 also carries the *unc-449* allele (Cox *et al.*, 1981), which results in the production of an altered α-subunit having a more acidic isoelectric point. The PBP present in A represents the small fraction (less than 5% of the total PBP) which is trapped within the membrane. Abbreviations: α and β, the α- and β-subunits, respectively, of the membrane-bound ATPase (Senior *et al.*, 1979); B, PstB protein; C, PstC protein; S, phosphate-binding protein (PBP); U, PhoU protein.

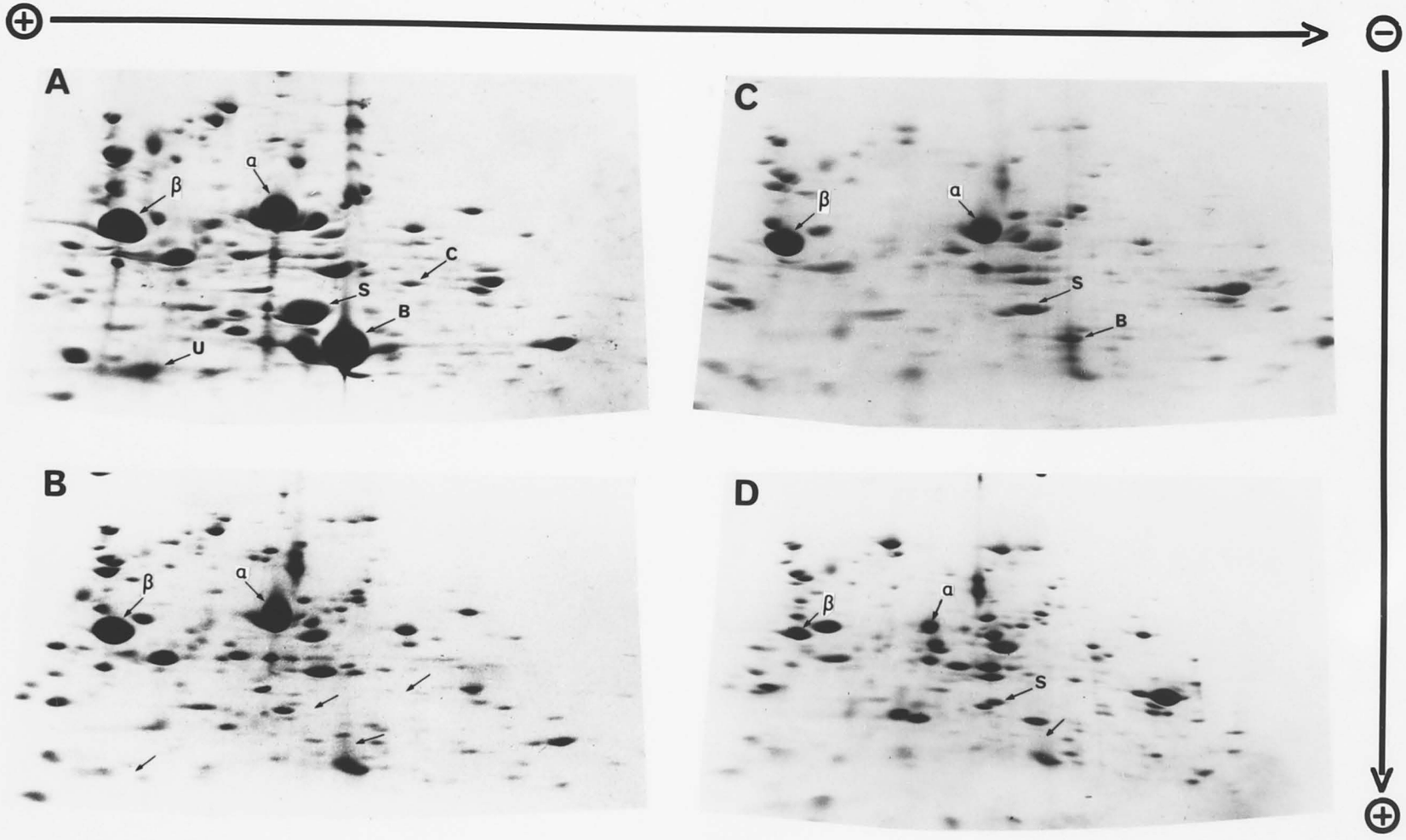
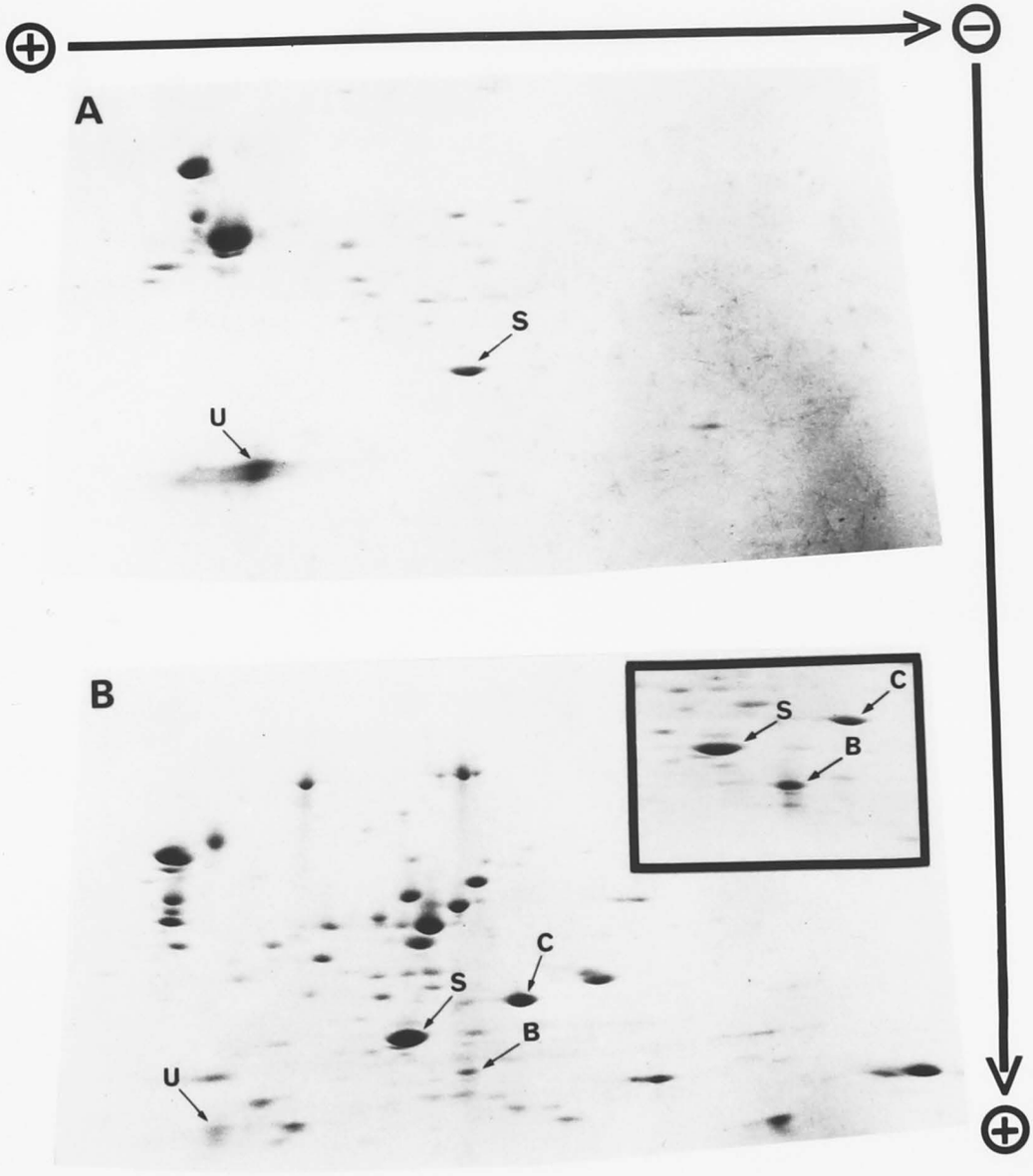

⊕
⊖
A
β
α
C
S
B
U
C
β
α
S
B
B
β
α
D
β
α
S
⊕

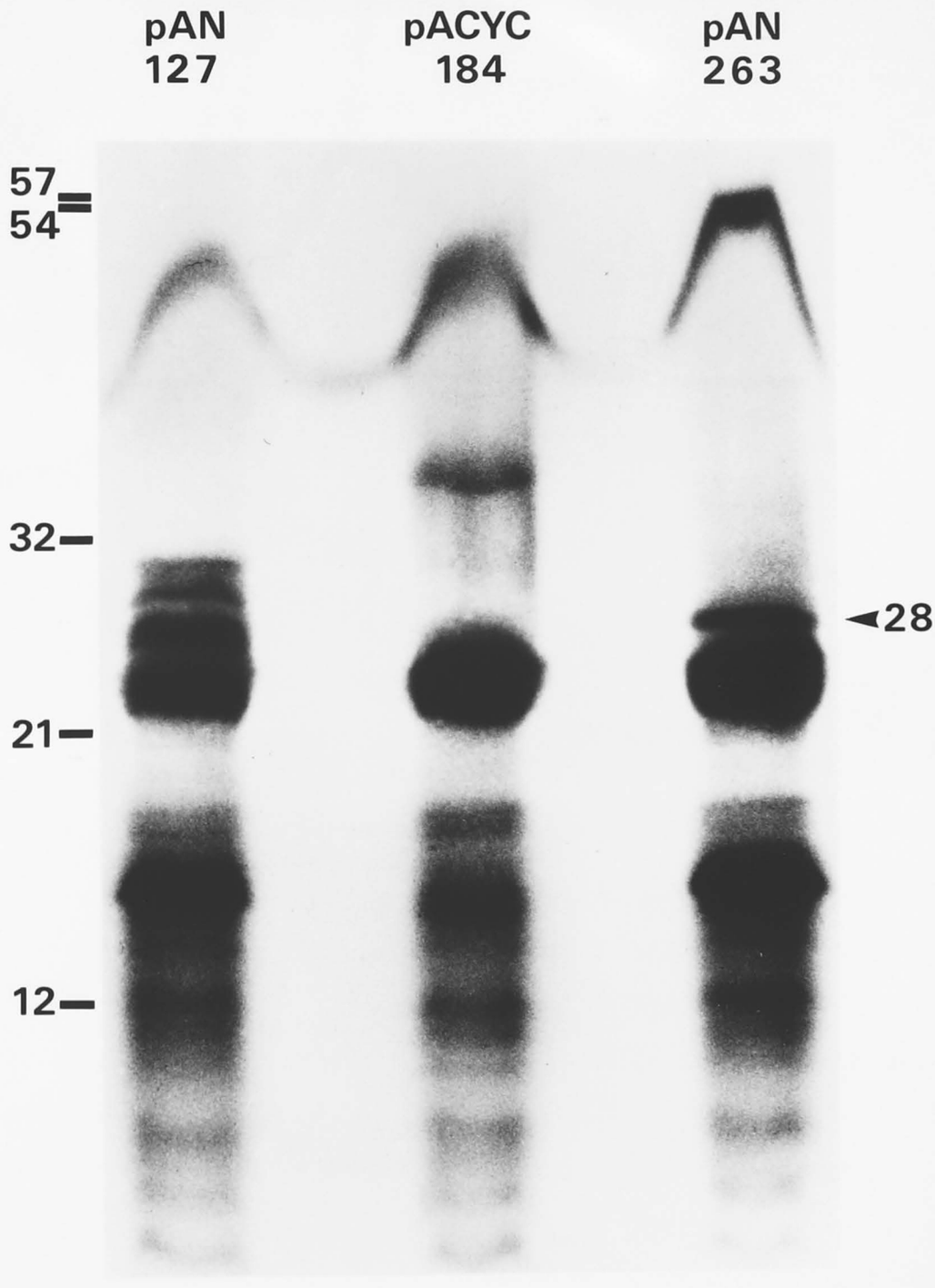
Fig. 4.5. Two-dimensional gel electrophoresis of proteins washed off the membrane fraction. Membranes were prepared from strain AN1667 (pAN92/*phoS*$^+$*pstC*$^+$*A*$^+$*B*$^+$*phoU*$^+$) grown under conditions of low Pi concentration (Chapter 2, Section 2.2E) and washed in low-ionic strength buffer in the presence of ρ-aminobenzamidine, followed by a 1M sodium phosphate or a 5M NaCl wash (Chapter 2, Section 2.3C). Two-dimensional gel electrophoresis was performed as described in Chapter 2 (Section 2.5C). Electrophoresis in the first dimension (isoelectric focusing) is in the horizontal direction, and in the second dimension (SDS-PAGE) in the vertical direction, of the figure. The labelled arrows refer to particular proteins identified by electrophoresis with authentic samples or with [^{35}S]methionine-labelled peptides derived from *in vitro* transcription/translation. A. Low-ionic strength wash of membranes. B. 1M sodium phosphate wash of membranes. Inset. A portion of the gel electrophoretogram of the 5M NaCl extraction of membranes. Extraction with 5M NaCl removes about 50% of the PstC and the PstB proteins from the membrane fraction. Abbreviations: B, PstB protein; C, PstC protein; S, phosphate-binding protein; U, PhoU protein.


A
S
U
B
C
S
B
C
S
B
U

dimensional gel electrophoretograms but was identified on one-dimensional SDS gel electrophoresis (Chapter 2, Section 2.7) and found to have a molecular weight of about 28,000 daltons (Fig. 4.6). The pstA gene product could not be detected in the subcellular fractions prepared from strain AN1667, regardless of the Pi concentration of the growth medium.

The pstB gene product was identified in the membrane fraction from strain AN1667 grown in media containing limiting concentrations of Pi as described above for the identification of the phoU gene product. The two-dimensional gel electrophoretogram (see Fig. 4.4A) revealed the presence of relatively large amounts of two proteins which were absent from membranes prepared from cells grown in excess Pi (Fig. 4.4B). One protein was readily identified by co-electrophoresis with purified PBP (Gerdes and Rosenberg, 1974). The second protein was present in membranes from phoU (Fig. 4.4C) or pstA (result not shown) mutant strains grown in media containing excess Pi but not in the pstB401 mutant grown under the same conditions (Fig. 4.4D). This protein was also formed in the in vitro transcription/translation system when plasmid pAN92 or plasmid pAN127 was used as DNA template (Fig. 4.3A and B), but was not formed from plasmid pAN271 (Fig. 4.3C) or plasmid pAN263 (result not shown). It was concluded that this protein, of about 29,000 daltons molecular weight and with an isoelectric point of about 6.5 is the product of the pstB gene. The PstB protein could not be removed from the membrane by low-ionic strength washing but was solubilized in the presence of 1M sodium phosphate or 5M NaCl (Fig. 4.5B).

Fig. 4.6. Autoradiograph of a gel showing the separation of [^{35}S]methionine-labelled peptides formed during *in vitro* transcription/translation. Plasmids pAN127 (*pstA*$^+$*B*$^+$*phoU*$^+$) and pAN263 (*pstA*$^+$) were constructed with plasmid pACYC184 as the vector. These plasmids were used as templates in an *in vitro* transcription/translation system. The reaction mixtures were then electrophoresed for 16h at 8mA using purified subunits of the F_1-ATPase as molecular weight markers (not shown). The numbers shown on the left-hand margin in the figure represent $M_r \times 10^{-3}$. The PstA protein was identified as a [^{35}S]methionine-labelled peptide having a molecular weight of about 28,000 daltons.


pAN
127
pACYC
184
pAN
263
57
54
32
21
12
28

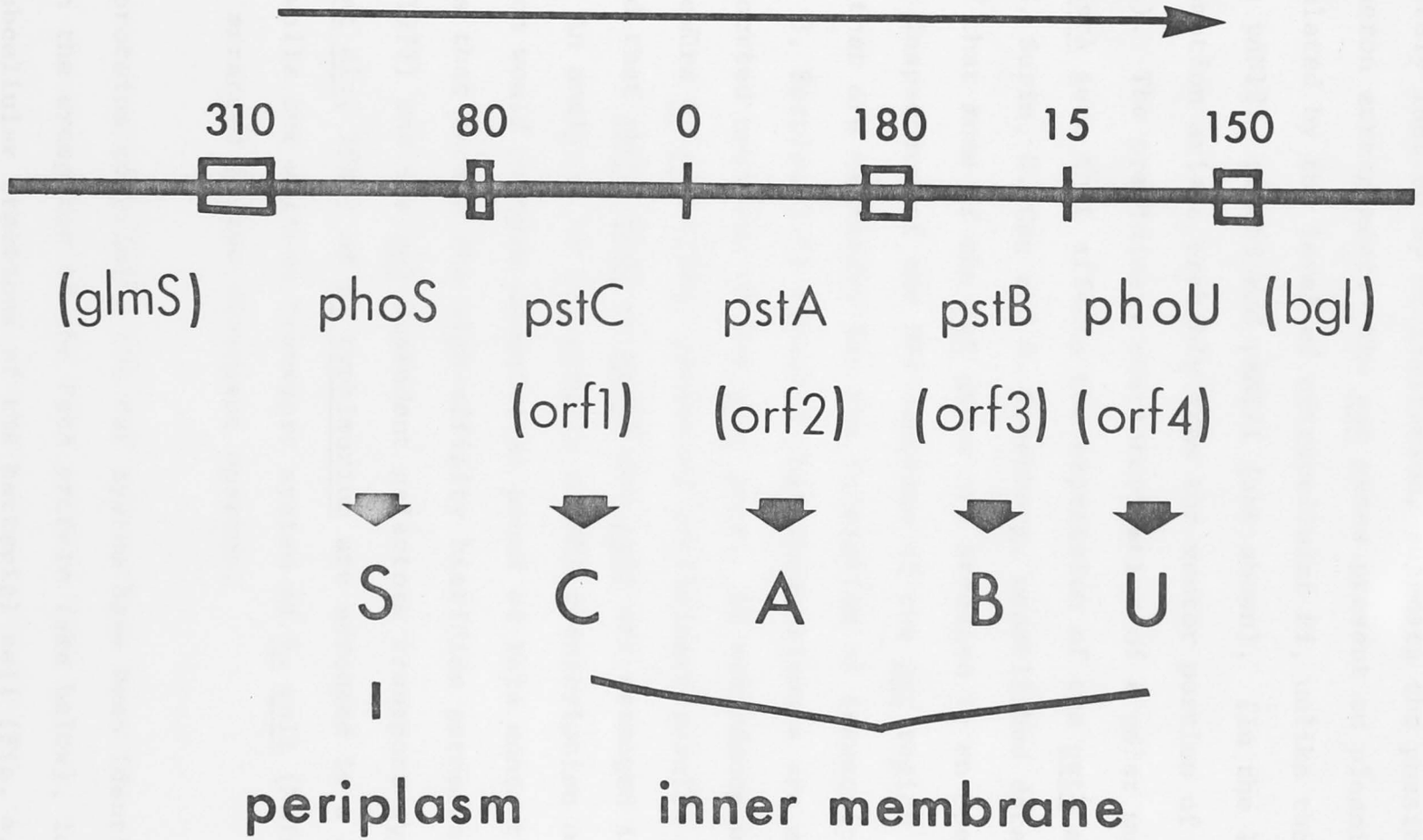
The product corresponding to the pstC gene was identified in the membrane fraction prepared from strain AN1667 grown in the low Pi minimal medium but was not present in cells grown in excess Pi (Fig. 4.4A and B). This protein, with a molecular weight of about 40,000 daltons and an isoelectric point of about 7.0, was solubilized with 1M sodium phosphate or 5M NaCl (Fig. 4.5B) and co-electrophoresed with a [^{35}S]methionine-labelled product from the in vitro transcription/translation system using plasmid pAN92 as the DNA template (Fig. 4.3A).

4.5 DISCUSSION

The components of the Pst system appear to be coded for by five genes, phoS, pstC, pstA, pstB, and phoU (Fig. 4.7), which are transcribed in a counterclockwise direction and located at about 83 min on the E. coli chromosome. The genetic organization of the pst region shown in Fig. 4.7 was confirmed by Amemura et al. (1985), with the exception that the authors used the mnemonic phoW for pstC. Levitz et al. (1984) reported the presence of a new locus, designated phoV, located downstream of the phoU gene. However this region is known to be essential for the regulation and initiation of transcription of the bgl operon (Reynolds et al., 1985), while the glmS gene is located upstream of the phoS gene (Walker et al., 1984). Thus I am unable to reconcile the report of Levitz et al. (1984) with my data, and with that of other groups (see above).

The observation that the genes encoding the Pst system are clustered together, transcribed in a common direction and

Fig. 4.7. The organization of the pst gene cluster. The structural genes including the intercistronic and regulatory regions (solid bar) are all drawn to scale. The two genes flanking the pst gene cluster are also shown. The horizontal arrow indicates the direction of transcription, which corresponds to the counterclockwise direction of the E. coli genetic map. Vertical lines or boxes on the solid bar delineate the extent of the pst genes. Numbers indicate the sizes of intergenic regions in base pairs. The proteins encoded by the pst genes and their subcellular locations are also shown.


310
80
0
180
15
150
(glmS)
phoS
pstC
pstA
pstB
phoU
(bgl)
(orf1)
(orf2)
(orf3)
(orf4)
S
C
A
B
U
periplasm
inner membrane

coordinately induced by Pi limitation, suggests the possibility of an operon arrangement. The pst genes present on plasmid pAN92 are regulated by the level of extracellular Pi, unlike those on plasmids pAN127, pAN263 and pAN271 (not shown). (In the latter case promotion arises probably from the vector portion of the plasmids). The preliminary characterization of a polar mutation in the pstA gene that affects the expression of the pstB and phoU genes (B. Surin, G. Cox and H. Rosenberg, unpublished data) suggests that some of the pst genes are arranged in an operon. Moreover inspection of the DNA sequence of the pst region for signals that are necessary for the initiation of transcription (Chapter 3, Section 3.6) revealed that these signals are most likely located upstream of the phoS gene. In accordance with this, Amemura et al. (1985) presented preliminary results which indicated that phoS, phoW (= pstC) and pstA are arranged in an operon. An analysis of the pattern of RNA transcription of the pst region would provide unequivocal proof of this contention. The genes that encode the high-affinity histidine permease (Ames et al., 1977) and the mgl-dependent galactose transport system (Muller et al., 1985) of S. typhimurium are arranged in a single operon, while the maltose transport system of E. coli (Hofnung, 1974) is arranged in two divergent operons.

The proteins comprising the Pst system have been identified and, with the exception of the PstA protein (see below), located in the subcellular fractions of the bacterial cell (Fig. 4.7). The PstC, PstB and PhoU proteins were identified in the inner membrane fraction (see Fig. 4.4). They were classified as peripheral proteins on the basis of solubilization without the

use of detergent. Thus the PhoU protein was extracted from the membrane by washing with the low-ionic strength buffer system, and the PstC and PstB proteins by 1M sodium phosphate. The PstC and the PhoU proteins were seen in trace amounts in the strain with a single copy of the pst genes, while the PBP and the PstB protein could be readily identified (Fig. 4.8). Relative to the periplasmic component (PBP) the inner membrane proteins of the Pst system are present in low amounts (see Fig. 4.4). A similar observation has been reported for the histidine permease of S. typhimurium (Higgins et al., 1982b) and the maltose transport system of E. coli (see Hengge and Boos, 1983).

The PBP is regulated by the level of extracellular Pi (Gerdes and Rosenberg, 1974). The PstC, PstB and PhoU proteins (see Fig. 4.4), and presumably the PstA protein, are also Pi-regulated. This accounts for the observation of Rosenberg et al. (1977) who reported that transport through the Pst system in whole cells was induced by Pi limitation, and that this induction was not seen in the presence of chloramphenicol, an inhibitor of protein synthesis.

The amino acid composition of the PstC, PstA, PstB and PhoU proteins, deduced from the nucleotide sequence (Chapter 3, Fig. 3.7) is presented in Table 4.2. The average hydropathic indices (Kyte and Doolittle, 1982) of these proteins show that the PstC and the PstA proteins are considerably more hydrophobic than the PstB and the PhoU proteins. The experimentally determined molecular weight and isoelectric point of each protein agrees, with some exceptions (see below), with the values expected from

Fig. 4.8. Comparison of the effect of gene dosage on the synthesis of Pst proteins *in vivo*. Shown here are the two-dimensional gel electrophoretograms of membranes (about 1mg) from strain AN1667 (pAN92/*phoS*$^+$*pstC*$^+$*A*$^+$*B*$^+$*phoU*$^+$) (A) and its non-plasmid containing isogenic sibling AN1664 (B). Both strains were grown in the low Pi minimal medium (Chapter 2, Section 2.2E). Two-dimensional gel electrophoresis was carried out as described in Chapter 2 (Section 2.5C). Electrophoresis in the first dimension (isoelectric focusing) is in the horizontal direction, and in the second dimension (SDS-PAGE) in the vertical direction, of the figure. The labelled arrows refer to particular proteins identified by electrophoresis with authentic samples or with [^{35}S]methionine-labelled peptides, derived from *in vitro* transcription/translation. The PstC and PhoU proteins in B are seen as very faint spots. Abbreviations: α and β, the α- and β-subunits, respectively, of the membrane-bound ATPase (Senior *et al.*, 1979); B, PstB protein; C, PstC protein; S, phosphate-binding protein; U, PhoU protein.

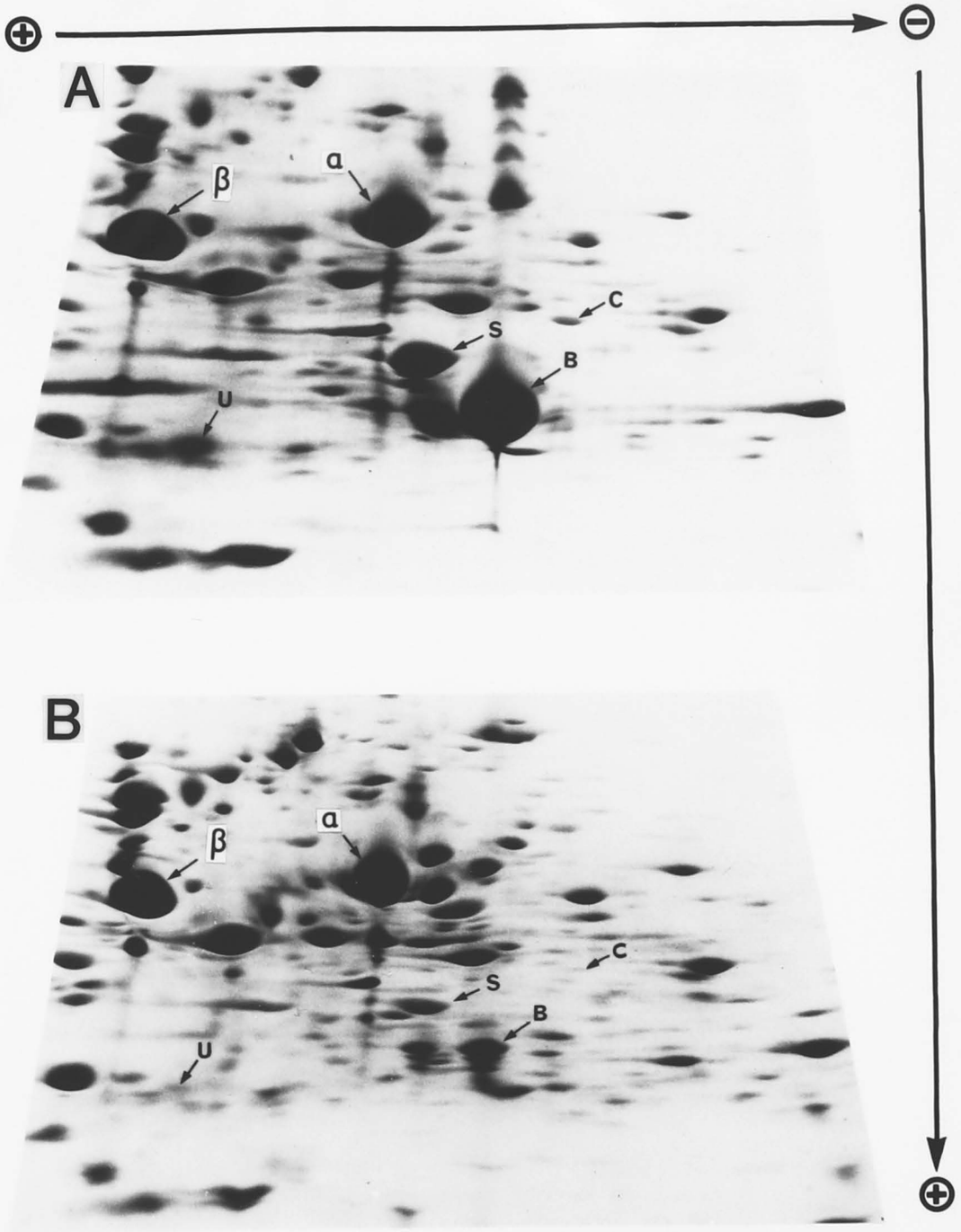

A
β
α
C
S
B
U
B
β
α
C
S
B
U

Table 4.2. Amino acid composition and other parameters of the PstC, PstA, PstB and PhoU proteins.

A. Amino acid composition.

Amino acid	Number of residues[a]			
	PstC	PstA	PstB	PhoU
Asn	9	9	12	7
Asp	5	3	15	22
Thr	14	22	17	10
Ser	17	16	14	14
Gln	4	8	14	14
Glu	13	11	16	18
Pro	18	12	10	3
Gly	31	21	16	12
Ala	38	38	19	17
Cys	1	1	4	5
Val	23	18	11	17
Met	14	17	8	13
Ile	40	27	18	20
Leu	39	41	27	21
Tyr	8	5	9	5
Phe	20	13	12	7
His	1	2	4	6
Lys	11	10	16	13
Arg	6	13	13	17
Trp	7	9	2	0
Total	319	296	257	241

.../contd. ...

Table 4.2. (/contd.)

B. Comparison of other parameters.

Parameter		PstC	PstA	PstB	PhoU
M_r	Calculated[a]	34,127	32,327	29,032	27,422
	Experimental[b]	40×10^3	28×10^3	29×10^3	26×10^3
Average hydropathic index[c]		0.99	0.71	-0.27	-0.27
Overall charge[d]		0	+11	+2	-4
Isoelectric point[b]		7.0	NK	6.5	5.4

[a] Determined from the corresponding nucleotide sequences (see Chapter 3).

[b] Determined from two-dimensional gel electrophoretograms with the subunits of the F_1F_0-ATPase as standards (Downie *et al.*, 1979a, 1979b; G. Cox, personal communication).

[c] Average hydropathic index was calculated according to Kyte and Doolittle (1982).

[d] Calculated assuming a charge of +1 for lys, arg and his, and a charge of -1 for asp and glu, at pH7.

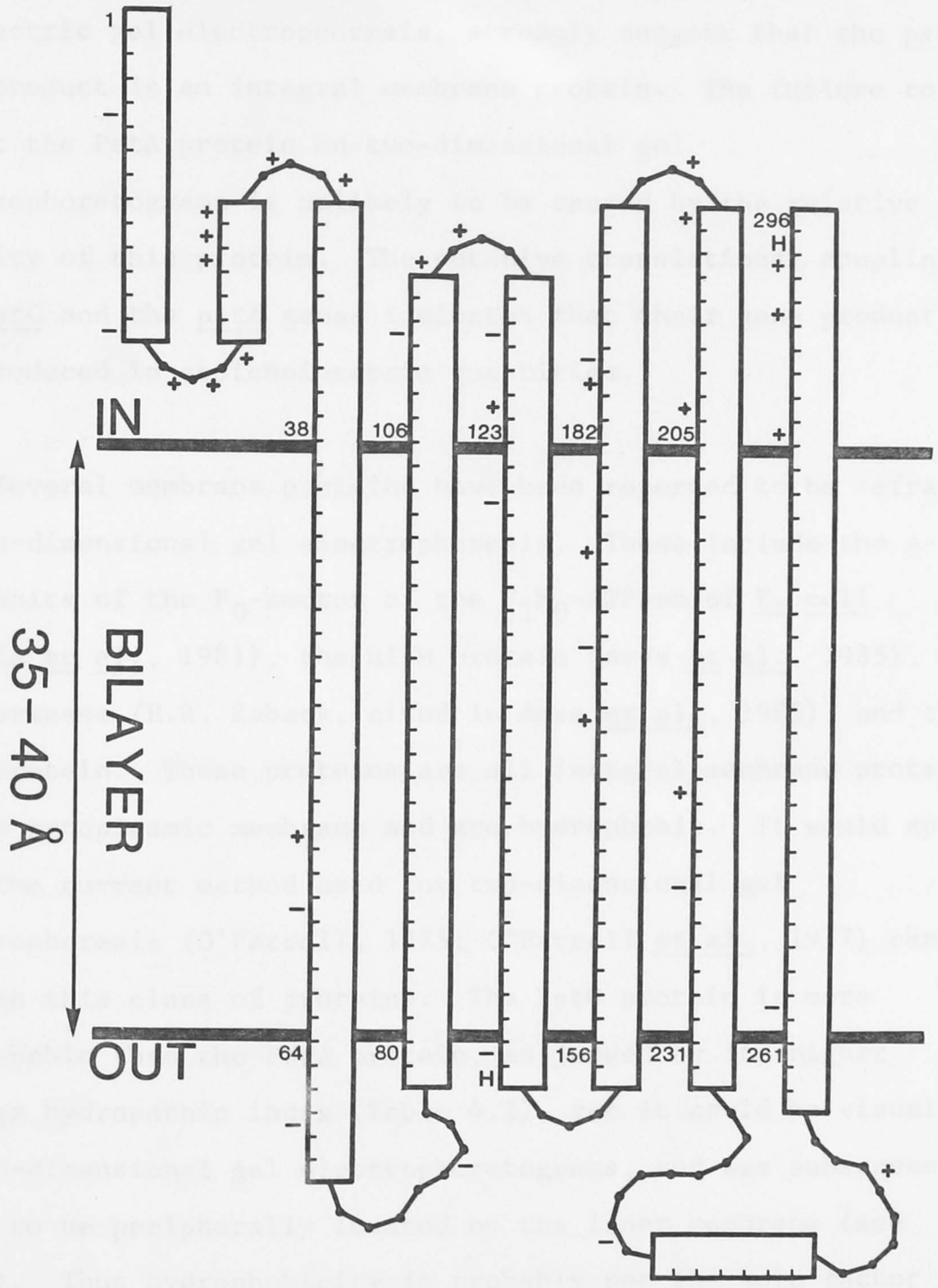
NK, Not known. This value could not be determined as the PstA protein does not focus during isoelectric gel electrophoresis.

the deduced amino acid sequence. This suggests that the reading frames identified within the nucleotide sequence in Chapter 3 are essentially correct. However the translational start-sites, especially where there is more than one alternative, must be regarded as tentative until confirmatory amino acid sequences of the corresponding proteins have been established.

The molecular weight of the PstC and the PstA proteins as determined experimentally by SDS-PAGE (see Section 4.4) differs by more than 10% from that predicted from the nucleotide sequence (Table 4.2). The unreliability of SDS-PAGE as an accurate determinant of molecular weight, particularly in the case of intrinsic membrane proteins, has been documented (Simon et al., 1977; Noel et al., 1979). The lactose permease, a highly hydrophobic inner membrane protein of E. coli, migrates during SDS-PAGE with an apparent molecular weight 13000 daltons lower than that calculated from the nucleotide sequence of the lacY gene (Newman et al., 1981).

The deduced amino acid composition of the pstA gene product (Table 4.2) shows that 13% of the residues in this protein are charged. The secondary and tertiary structure of the PstA protein was predicted according to Chou and Fasman (1978), and is depicted in Fig. 4.9. This structure consists of six transmembrane α-helices arranged perpendicularly to the plane of the membrane, in an analogous manner to the structure deduced for bacteriorhodopsin from electron microscopy and electron diffraction studies (Henderson and Unwin, 1975). This structure, coupled with the observed failure of this protein to focus during

Fig. 4.9. Predicted secondary and tertiary structure of the PstA protein. The method of prediction is that of Chou and Fasman (1978). The secondary structures are as indicated:  α- helices;  β-turns;  (in random configuraton) random coil. It is assumed that one residue in β-configuration occupies the space equivalent to two residues in α-helical configuration. Charged residues are as indicated, while H denotes a histidine residue. The molecule is 84% α-helical.


1
IN
38
106
123
182
205
296
H
35-40 Å
BILAYER
OUT
64
80
H
156
231
261

isoelectric gel electrophoresis, strongly suggest that the pstA gene product is an integral membrane protein. The failure to detect the PstA protein on two-dimensional gel electrophoretograms is unlikely to be caused by the relative scarcity of this protein. The putative translational coupling of the pstC and the pstA genes indicates that their gene products are produced in stoicheiometric quantities.

Several membrane proteins have been reported to be refractory to two-dimensional gel electrophoresis. These include the a- and c-subunits of the F_0-sector of the F_1F_0-ATPase of E. coli (Downie et al., 1981), the HisM protein (Ames et al., 1985), the lac permease (H.R. Kaback, cited in Ames et al., 1985), and the PstA protein. These proteins are all integral membrane proteins of the cytoplasmic membrane and are hydrophobic. It would appear that the current method used for two-dimensional gel electrophoresis (O'Farrell, 1975; O'Farrell et al., 1977) cannot resolve this class of proteins. The PstC protein is more hydrophobic than the PstA protein, as judged by its higher average hydropathic index (Table 4.2), yet it could be visualized on two-dimensional gel electrophoretograms, and was subsequently shown to be peripherally located on the inner membrane (see above). Thus hydrophobicity is probably not the sole factor which determines whether a protein can be resolved by two-dimensional gel electrophoresis. Other factors, including the degree of interaction with the phospholipids of the inner membrane, may be important.

It was suggested earlier that the phoU35 allele does not affect Pi transport through the Pst system (Zuckier and Torriani, 1981), and this result is confirmed here (Table 4.1). The rate of Pi uptake through the Pst system in the phoU35 mutant is about twice that of a fully induced wild strain (H. Rosenberg, personal communication), and does not require a prior period of Pi starvation: it is constitutive. Consistent with this is the observation that the PBP and the PstB protein, the only components of the Pst system that can be readily visualized when the pst genes are in single-copy (see Fig. 4.8), are synthesized during growth in the presence of excess Pi in this mutant (see Fig. 4.4C).

The pst gene cluster consists of five components, one more than the number noted for all other shock-sensitive transport systems (Chapter 1, Section 1.6). The pst gene for which there is no correspondent in other shock-sensitive transport systems is phoU, which encodes a protein that is not essential for Pi transport (see Chapter 6), but whose presence may be related to the postulated role of the Pst system in the regulation of gene expression in the pho regulon (see Chapter 8). The Pst system, the high-affinity histidine permease and the maltose transport system have a similar composition of inner membrane proteins. The histidine permease of S. typhimurium has two hydrophobic inner membrane proteins (HisQ and HisM) and a third inner membrane protein, HisP, which is hydrophilic (Ames and Nikaido, 1978; Higgins et al., 1982b; Ames et al., 1985). In the maltose transport system two proteins located in the inner membrane, MalF (Froshauer and Beckwith, 1984) and MalG (Dassa and Hofnung,

1985), have been found to be hydrophobic, while a third inner membrane protein (MalK) has been shown to be hydrophilic (Gilson et al., 1982b).

A conserved segment of amino acids located near the C-termini of the hydrophobic proteins of these systems has been described (Dassa and Hofnung, 1985). This homology may simply reflect a common evolutionary origin, or it may indicate a common step (for example, protein-protein interaction) in substrate translocation by these systems. Extensive amino acid sequence homologies have been documented for the hydrophilic components, the PstB, HisP and MalK proteins, of these systems, and for the OppD protein of the oligopeptide permease of S. typhimurium. A comparison of a large number of adenine nucleotide-binding proteins has led to the identification of a consensus amino acid sequence required to form an adenine nucleotide-binding fold (Walker et al., 1982). This putative adenine nucleotide-binding fold consists of two sequence blocks (Fig. 4.10). There are two regions present in the OppD, HisP, MalK and PstB proteins in which the amino acid sequence is highly conserved (Fig. 4.10) (Higgins et al., 1985; C.F. Higgins, personal communication). Significantly the two sequence blocks which comprise the putative adenine nucleotide-binding fold are located within the two highly conserved regions of these proteins (Fig. 4.10).

An ATP-binding site has been experimentally demonstrated for the HisP (Hobson et al., 1984) and the OppD proteins (Higgins et al., 1985). Berger and Heppel (1974) originally proposed, on the basis of studies of the energetics of active transport in whole

Fig. 4.10. Consensus adenine nucleotide-binding sequences. The OppD, HisP, MalK and PstB proteins are aligned with several other adenine nucleotide-binding proteins to illustrate the two sequence blocks (A and B) which comprise the consensus adenine nucleotide-binding fold. Regions of particular amino acid homology, including conservative substitutions, are boxed. * in the consensus sequence indicates a conserved hydrophobic residue. All amino acid sequences (except the PstB protein), their alignment and the identification of a consensus adenine nucleotide-binding sequence are from Walker et al. (1982) and Higgins et al. (1985). The identification of a consensus adenine nucleotide-binding fold in the PstB protein was performed by C.F. Higgins (personal communication).

(A)

PROTEIN	RESIDUES	SEQUENCE
Bovine ATPase β	149-168	K G G K I G L F - G G A G V G K T - V F I M
E. coli ATPase β	142-161	K G G K V G L F - G G A G V G K T - V N M M
E. coli ATPase α	161-180	R G Q R E L I I - G D R G T G K T - A L A I
Adenylate Kinase	6- 26	K K S K I I F V V G G P G S G K G - T Q C E
OppD protein	46- 66	A G E T L G I V - G E S G S G K S Q S R L R
HisP protein	31- 50	A G D V I S I I - G S S G S G K S - T F L R
MalK protein	28- 47	E G G F V V F V - G P S G C G K S - T L L R
PstB protein	35- 54	K N Q V T A F I - G P S G C G K S - T L L R
CONSENSUS		G G G K S/T

(B)

PROTEIN	RESIDUES	SEQUENCE
Bovine ATPase β	241-267	V A E Y F R D Q E G Q D V L L F I D N I F R F T Q A G
E. coli ATPase β	227-252	M A E K F R D - E G R D V L L F V D N I Y R Y T L A G
E. coli ATPase α	265-290	M G E Y F R D - R G E D A L I I Y D D L S K Q A V A Y
Adenylate Kinase	102-127	G E E F E R K - I G Q P T L L L Y V D A G P E T M T K
OppD protein	173-200	Q R V M I A M A L L C R P K L L I A D E P T T A L D V T
HisP protein	160-187	Q R V S I A R A L A M E P D V L L F D E P T S A L D P E
MalK protein	140-167	Q R V A I G R T L V A E P S V F L L D E P L S N L D A A
PstB protein	160-187	Q R L C I A R G I A I R P E V L L L D E P C S A L D P I
CONSENSUS		R * * * * D D/E

cells, that shock-sensitive transport systems were energized by direct hydrolysis of ATP or a closely related metabolite. It has been suggested (Hengge and Boos, 1983) that these systems function by a common mechanism involving conformational changes induced by binding and hydrolysis of ATP, perhaps in a manner analogous to the ATP-driven ion pumps (Tanford, 1983). This proposal is supported by the observed similarities which such systems manifest with regard to the mode of energization of solute transport (see Chapter 1, Sections 1.3C2 and 1.4B). In the model of Hengge and Boos (1983) the PstB, HisP, MalK and OppD proteins would be the likely component involved in the coupling of energy to active transport in their respective systems. The use of inhibitors that bind in a specific manner to the nucleotide-binding sites of these proteins will enable this proposal to be tested. Preliminary experiments of this nature have been attempted (Richarme, 1985). However the possibility remains that nucleotide-binding simply plays a regulatory role. More evidence is needed to prove that hydrolysis of ATP or a related metabolite energizes solute transport in these systems.

CHAPTER 5

ROLE OF THE pstC GENE IN THE PST SYSTEM

5.1 INTRODUCTION

The elucidation of the complete nucleotide sequence of the pst region revealed the presence of five genes: phoS, pstC, pstA, pstB, and phoU. The role of the phoS, pstA (= phoT), pstB and phoU genes in the regulation of alkaline phosphatase synthesis and in the uptake of Pi has been reported (Chapter 1, Sections 1.4A and 1.4C1). The pstA and pstC genes each encode a hydrophobic protein located in the inner membrane. The pstC gene had not been described hitherto and no mutations mapping in this gene were known. The shock-sensitive histidine permease of S. typhimurium (Higgins et al., 1982b) and the maltose transport system of E. coli (Froshauer and Beckwith, 1984; Dassa and Hofnung, 1985) have two hydrophobic proteins located in the inner membrane whose activity is essential for substrate translocation.

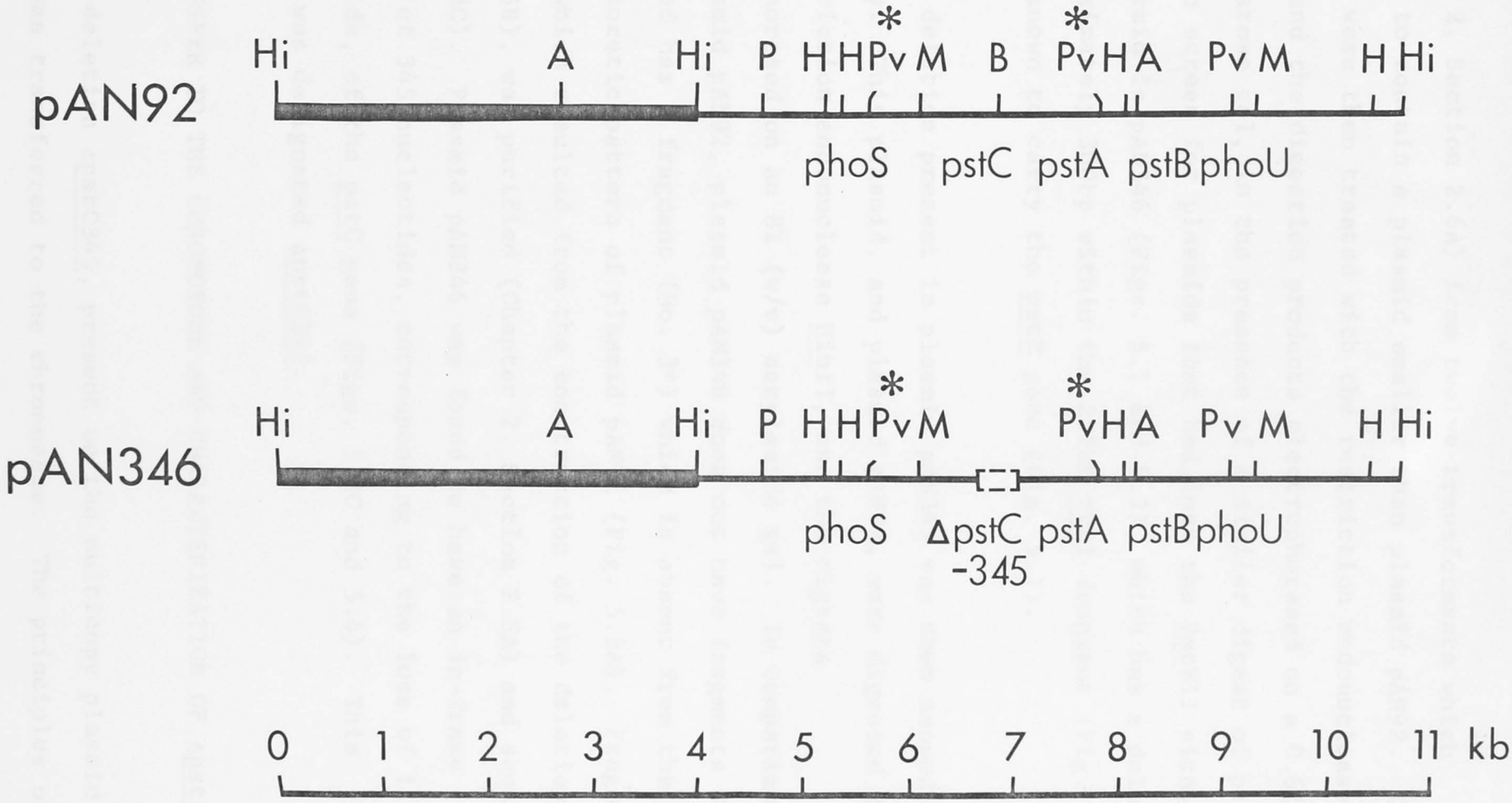
In order to study the role of the pstC gene in the Pst system, I constructed a strain with a pstC deletion on the chromosome and investigated its phenotype with respect to high-affinity Pi transport and alkaline phosphatase activity.

5.2 CONSTRUCTION OF A DELETION WITHIN THE *pstC* GENE

The *pstC* gene has within its protein-coding sequence a site for the restriction endonuclease *Bst*EII (Fig. 5.1). This site, which is unique in plasmid pAN92, was used to construct a deletion within the *pstC* gene. Plasmid pAN92 was digested with the restriction endonuclease *Bst*EII, and the linearized plasmid treated with the nuclease *Bal*31 (Chapter 2, Section 2.6D). This nuclease progressively degrades the ends of both strands of double-stranded DNA (Gray *et al.*, 1975). The digestion with the nuclease *Bal*31 was quenched at different time intervals after the addition of the enzyme, and the quenched reaction mixtures were electrophoresed on a 0.6% (w/v) agarose gel. DNA fragments possessing deletions of up to 500 base pairs (bp), and which appeared to be present in the incubations quenched after 10, 15 and 20 minutes, were extracted from the agarose gel as described in Chapter 2 (Section 2.6J). The purified DNA fragments were then ligated, and incubated with the restriction endonuclease *Bst*EII, before transformation into strain AN1459 (*ilvC thr leu recA*). Since linear plasmids transform with much lower efficiency than circular plasmids (Conley and Saunders, 1984), the *Bst*EII digestion was included before transformation to linearize plasmids that had not lost the *Bst*EII site and thus ensure a lower recovery of such plasmids after transformation.

Transformants were selected for resistance to chloramphenicol on rich medium. Single transformants were screened for the presence and size of recombinant plasmids by the plasmid sizing procedure (Chapter 2, Section 2.6H). Plasmid DNA was prepared

Fig. 5.1. Physical map and derivation of plasmid pAN346. Plasmid pAN92 was digested with the restriction endonuclease BstEII, and the linearized plasmid treated with the nuclease Bal31. After purification of DNA fragments and ligation, as described in the text (Section 5.2), plasmid pAN346 was obtained. The approximate locations of genes on the various plasmids are as shown. Plasmid pAN346 carries ΔpstC345, a 345 nucleotide deletion of the pstC gene. The broken box represents the deletion, while the heavy line indicates the vector (plasmid pACYC184) portion of the plasmids. Asterisks denote the PvuI restriction endonuclease-generated fragment which carries the wild type pstC gene in plasmid pAN92, and ΔpstC345 in plasmid pAN346 (see Fig. 5.2). Restriction endonuclease sites: A, AvaI; B, BstEII; H, HpaI; Hi, HindIII; M, MluI; P, PstI; Pv, PvuI. kb, kilobase pair.


pAN92
Hi
A
Hi
P
H
H
Pv
M
B
Pv
H
A
Pv
M
H
Hi
*
*
phoS
pstC
pstA
pstB
phoU
pAN346
Hi
A
Hi
P
H
H
Pv
M
Pv
H
A
Pv
M
H
Hi
*
*
phoS
ΔpstC
-345
pstA
pstB
phoU
0
1
2
3
4
5
6
7
8
9
10
11
kb

(Chapter 2, Section 2.6A) from twelve transformants which appeared to contain a plasmid smaller than plasmid pAN92. These plasmids were then treated with the restriction endonuclease BstEII, and the digestion products electrophoresed on a 0.6% (w/v) agarose gel, in the presence of a similar digest of plasmid pAN92, to screen for plasmids that had lost the BstEII site. One such plasmid is pAN346 (Figs. 5.1 and 5.2), which has a deletion of approximately 300bp within the 2.2kb PvuI fragment (Fig. 5.2) that is known to carry the pstC gene (Fig. 5.1).

The deletion present in plasmid pAN346 was then mapped more precisely. This plasmid, and plasmid pAN92, were digested with the restriction endonuclease HinfI, and the digests electrophoresed on an 8% (w/v) acrylamide gel. In comparison with plasmid pAN92, plasmid pAN346 does not have fragments No. 3 and 14 and has a fragment (No. 3*) which is absent from the electrophoretic pattern of plasmid pAN92 (Fig. 5.3A). Fragment No. 3*, which resulted from the construction of the deletion (Fig. 5.3B), was purified (Chapter 2, Section 2.8A) and sequenced (Fig. 5.3C). Plasmid pAN346 was found to have an in-frame deletion of 345 nucleotides, corresponding to the loss of 115 amino acids, of the pstC gene (Figs. 5.3C and 5.4). This deletion was designated ΔpstC345.

5.3 TRANSFER TO THE CHROMOSOME AND CHARACTERIZATION OF ΔpstC345

The deletion ΔpstC345, present on the multicopy plasmid pAN346, was transferred to the chromosome. The principles of this procedure have been described (Gutterson and Koshland, 1983;

Fig. 5.2. Agarose gel electrophoresis of plasmid DNA. Plasmid DNA was digested with restriction endonuclease (see below) and electrophoresed on an agarose gel (0.6%, w/v), in the presence of λ DNA digested with the restriction endonuclease HindIII as size marker. (A), (E) and (H) λ DNA digested with HindIII; (B) pAN92 digested with BstEII; (C) pAN346 digested with PstI; (D) pAN346 digested with BstEII; (F) pAN92 digested with PvuI; (G) pAN346 digested with PvuI. In (D) plasmid pAN346 migrates as closed circular DNA, indicating that the unique BstEII restriction endonuclease site present in plasmid pAN92 (see B) is absent. The 2.2kb PvuI fragment of plasmid pAN92 carries all of the pstC gene (see Fig. 5.1). In plasmid pAN346 this fragment (indicated with an arrowhead) is approximately 300 base pairs smaller, verifying the presence of the deletion ΔpstC345.

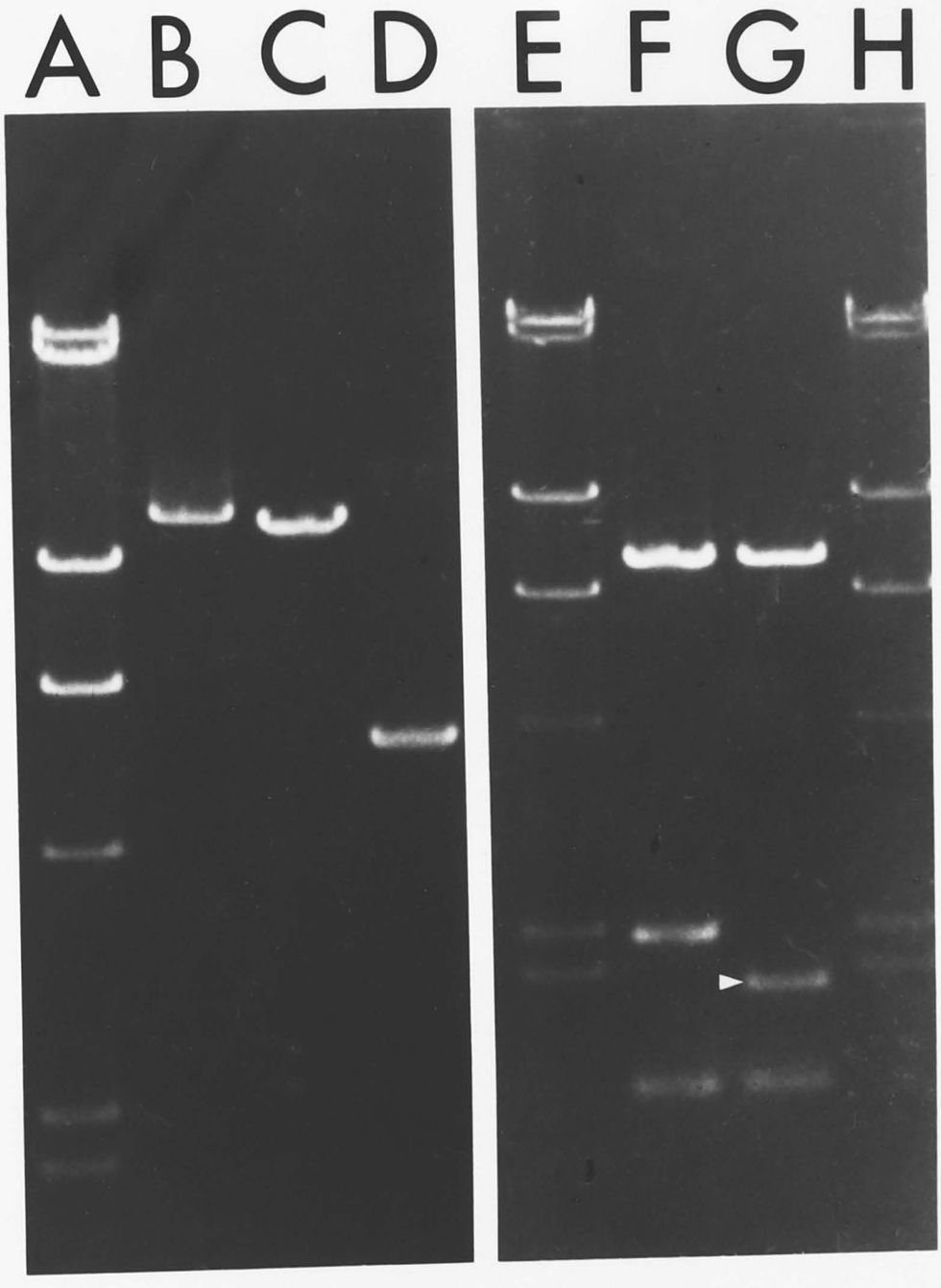

A B C D E F G H

Fig. 5.3. A. Polyacrylamide gel electrophoresis of HinfI restriction endonuclease cleavage fragments of plasmid DNA. B. HinfI restriction fragment map of plasmid DNA adjacent to and including the deletion ΔpstC345. C. Section of a DNA sequencing gel from plasmid pAN346. In A samples were run on an 8% (w/v) acrylamide gel as described in Chapter 2 (Section 2.6I). (1) pAN92 digested with HinfI; (2) pAN346 digested with HinfI. The arrowheads in the electrophoretic pattern of the pAN92 digest indicate the restriction fragments (3 and 14) which are absent from the pattern for plasmid pAN346. The arrowhead in the electrophoretic pattern for plasmid pAN346 indicates a single HinfI restriction endonuclease-generated fragment (3*) which is absent from plasmid pAN92. In B vertical arrowheads indicate HinfI restriction endonuclease sites. The complete HinfI restriction fragment map of plasmid pAN92 is presented in Chapter 3 (Fig. 3.5). Plasmid pAN346 was derived from plasmid pAN92 by deletion of the region as shown in the figure. In C a section of a 6% (w/v) polyacrylamide sequencing gel of fragment No. 3* (see above) is shown. The fragment was end-filled with [α-^{32}P]dATP as the source of radioactivity, and the labelled-ends separated by digestion with the restriction endonuclease AluI. The arrowhead indicates the point from which the 345 nucleotides have been deleted.

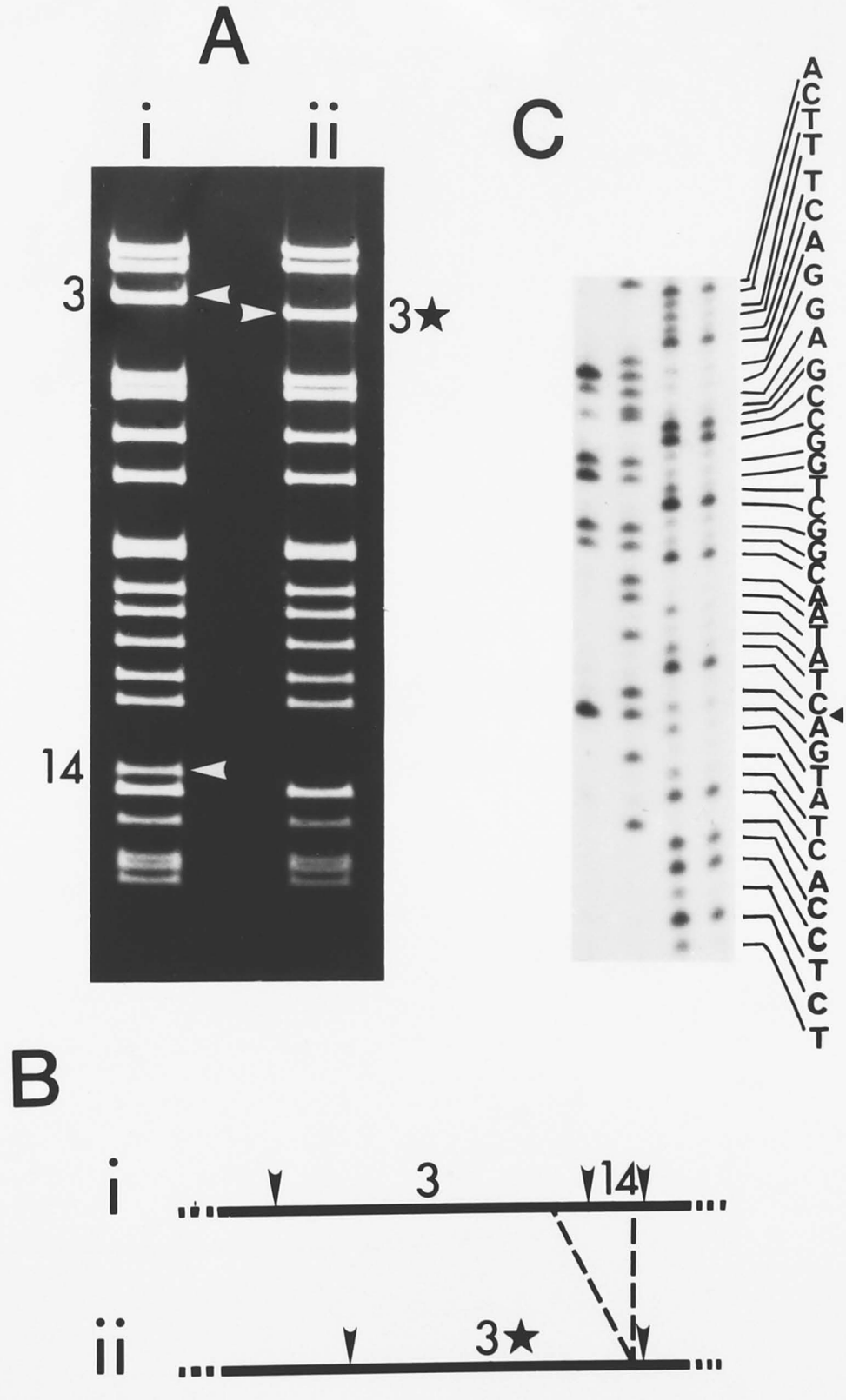
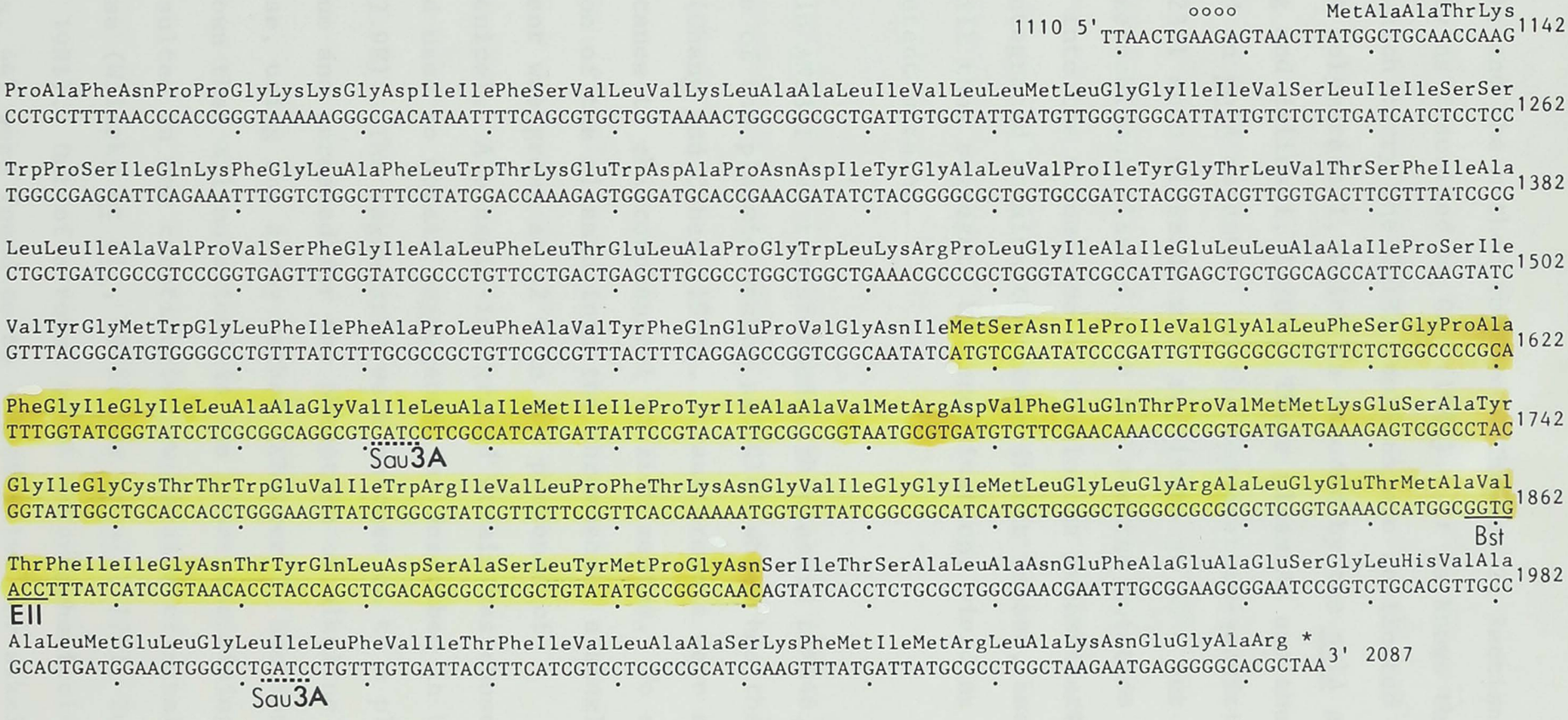

A
i
ii
3
3★
14
C
ACTTTCAGGAGCCCGGTCGCCAATATCAGTATCACCCTCT
B
i
3
14
ii
3★

Fig. 5.4. Nucleotide sequence of the pstC gene, including the deletion ΔpstC345, and the corresponding amino acid sequence. Nucleotides representing part of the putative ribosome-binding site are indicated by an open circle, while an asterisk denotes the proposed translational stop codon (TAA). The nucleotides absent in ΔpstC345 are indicated by the yellow colour. The DNA recognition sequence (GGTNACC) of the restriction endonuclease BstEII is indicated. The DNA fragment that is enclosed within the Sau3A restriction sites (GATC) shown on the figure was used as a probe to screen for the presence of ΔpstC345 on the chromosome (see Section 5.3).


oooo
MetAlaAlaThrLys
1110 5' TTAACTGAAGAGTAACTTATGGCTGCAACCAAG 1142
ProAlaPheAsnProProGlyLysLysGlyAspIleIlePheSerValLeuValLysLeuAlaAlaLeuIleValLeuLeuMetLeuGlyGlyIleIleValSerLeuIleIleSerSer
CCTGCTTTTAACCCACCGGGTAAAAAGGGCGACATAATTTTCAGCGTGCTGGTAAAACTGGCGGCGCTGATTGTGCTATTGATGTTGGGTGGCATTATTGTCTCTCTGATCATCTCCTCC 1262
TrpProSerIleGlnLysPheGlyLeuAlaPheLeuTrpThrLysGluTrpAspAlaProAsnAspIleTyrGlyAlaLeuValProIleTyrGlyThrLeuValThrSerPheIleAla
TGGCCGAGCATTCAGAAATTTGGTCTGGCTTTCCTATGGACCAAAGAGTGGGATGCACCGAACGATATCTACGGGGCGCTGGTGCCGATCTACGGTACGTTGGTGACTTCGTTTATCGCG 1382
LeuLeuIleAlaValProValSerPheGlyIleAlaLeuPheLeuThrGluLeuAlaProGlyTrpLeuLysArgProLeuGlyIleAlaIleGluLeuLeuAlaAlaIleProSerIle
CTGCTGATCGCCGTCCCGGTGAGTTTCGGTATCGCCCTGTTCCTGACTGAGCTTGCGCCTGGCTGGCTGAAACGCCCGCTGGGTATCGCCATTGAGCTGCTGGCAGCCATTCCAAGTATC 1502
ValTyrGlyMetTrpGlyLeuPheIlePheAlaProLeuPheAlaValTyrPheGlnGluProValGlyAsnIleMetSerAsnIleProIleValGlyAlaLeuPheSerGlyProAla
GTTTACGGCATGTGGGGCCTGTTTATCTTTGCGCCGCTGTTCGCCGTTTACTTTCAGGAGCCGGTCGGCAATATCATGTCGAATATCCCGATTGTTGGCGCGCTGTTCTCTGGCCCCGCA 1622
PheGlyIleGlyIleLeuAlaAlaGlyValIleLeuAlaIleMetIleIleProTyrIleAlaAlaValMetArgAspValPheGluGlnThrProValMetMetLysGluSerAlaTyr
TTTGGTATCGGTATCCTCGCGGCAGGCGTGATCCTCGCCATCATGATTATTCCGTACATTGCGGCGGTAATGCGTGATGTGTTCGAACAAACCCCGGTGATGATGAAAGAGTCGGCCTAC 1742
Sau3A
GlyIleGlyCysThrThrTrpGluValIleTrpArgIleValLeuProPheThrLysAsnGlyValIleGlyGlyIleMetLeuGlyLeuGlyArgAlaLeuGlyGluThrMetAlaVal
GGTATTGGCTGCACCACCTGGGAAGTTATCTGGCGTATCGTTCTTCCGTTCACCAAAAATGGTGTTATCGGCGGCATCATGCTGGGGCTGGGCCGCGCGCTCGGTGAAACCATGGCGGTG 1862
Bst
ThrPheIleIleGlyAsnThrTyrGlnLeuAspSerAlaSerLeuTyrMetProGlyAsnSerIleThrSerAlaLeuAlaAsnGluPheAlaGluAlaGluSerGlyLeuHisValAla
ACCTTTATCATCGGTAACACCTACCAGCTCGACAGCGCCTCGCTGTATATGCCGGGCAACAGTATCACCTCTGCGCTGGCGAACGAATTTGCGGAAGCGGAATCCGGTCTGCACGTTGCC 1982
EII
AlaLeuMetGluLeuGlyLeuIleLeuPheValIleThrPheIleValLeuAlaAlaSerLysPheMetIleMetArgLeuAlaLysAsnGluGlyAlaArg *
GCACTGATGGAACTGGGCCTGATCCTGTTTGTGATTACCTTCATCGTCCTCGCCGCATCGAAGTTTATGATTATGCGCCTGGCTAAGAATGAGGGGGCACGCTAA 3' 2087
Sau3A

Gay, 1984), and the method employed (Chapter 2, Section 2.9E) is essentially as described by Gay (1984). It is known that plasmids which carry the ColE1 replicon are replicated by the enzyme DNA polymerase I, which is encoded by the polA gene (Kingsburg and Helinski, 1970). These plasmids are thus not replicated in polA mutants. In the temperature-sensitive polA mutant AN2351 such plasmids will replicate at 30°C but not at 42°C. A recombinant plasmid with the desired mutation may integrate into the chromosome by homologous recombination. Such plasmid-integrated strains contain a direct repeat, and the plasmid will thus segregate by recombination, yielding either wild or mutant strains.

Strain AN2351 was transformed with plasmid pAN346, a derivative of the plasmid vector pACYC184 which has the ColE1 replicon (Chang and Cohen, 1978). Transformants were selected for resistance to chloramphenicol on rich medium. To select for integration of the plasmid into the chromosome, a single transformant was grown at 42°C in the presence of chloramphenicol. A plasmid-integrated strain was chosen at random and used to obtain segregants, as described in Chapter 2 (Section 2.9E). The segregants were patched on to a plate of rich medium and screened for the phenotype of alkaline phosphatase, using the spray method (Chapter 2, Section 2.4A). It was known that all mutations in the genes comprising the Pst system resulted in the constitutive expression of alkaline phosphatase (Willsky et al., 1973; Cox et al., 1981; Zuckier and Torriani, 1981). Out of a total of 88 chloramphenicol-sensitive segregants, 44% were found to be constitutive for alkaline

phosphatase synthesis. In contrast all the segregants obtained from strain AN2351 transformed with the vector plasmid pACYC184 expressed a phosphate-repressible alkaline phosphatase phenotype.

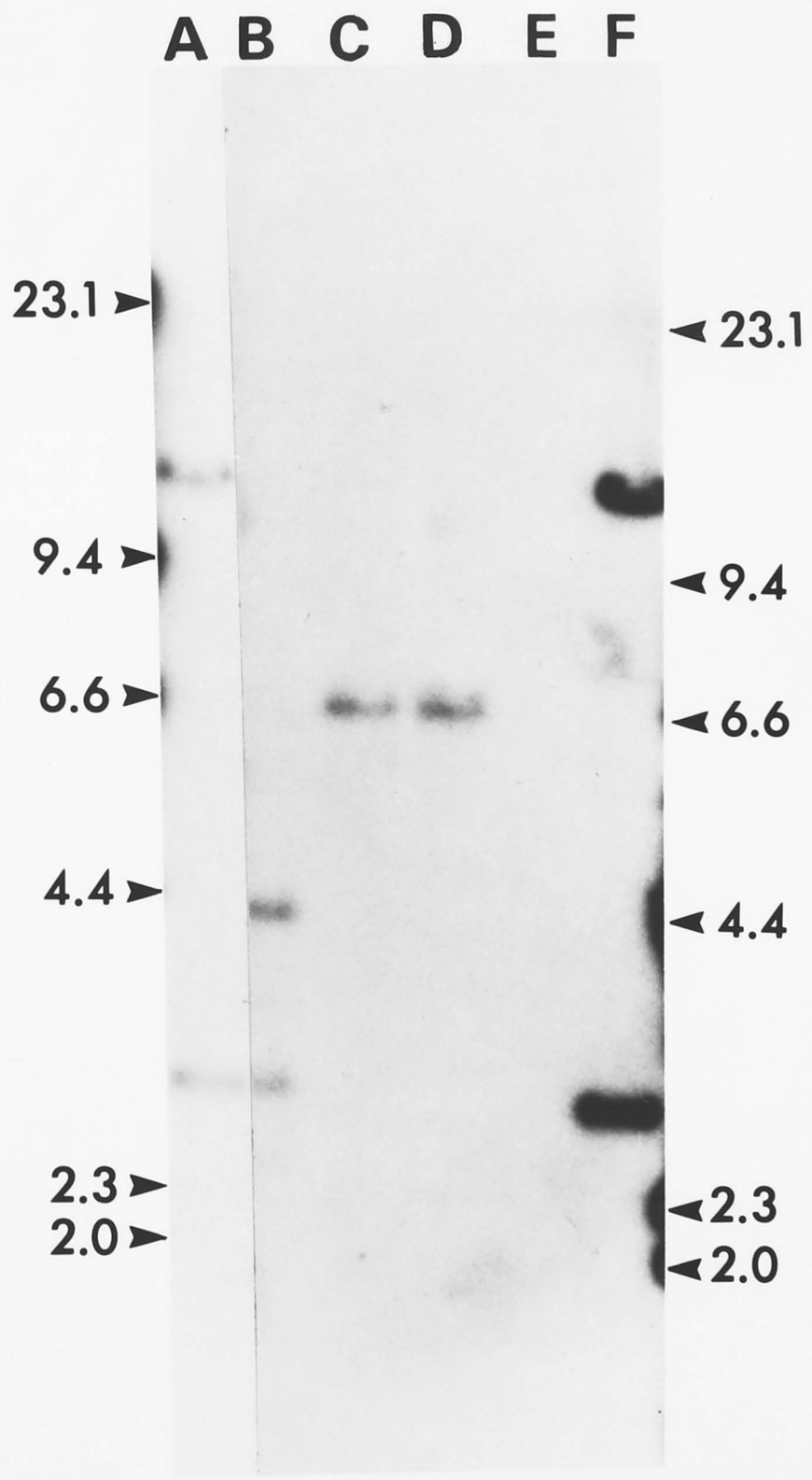
One of the segregants which expressed an alkaline phosphatase constitutive phenotype was used as a donor for bacteriophage P1-mediated transduction (Chapter 2, Section 2.9B). A bacteriophage P1 lysate was prepared from this segregant and used to transduce the alkaline phosphatase constitutive phenotype with the *ilv* genes into the female strain AN346 (*ilv*). This phenotype was found to be 56% cotransducible with *ilv*. Since the *pst* region is about 60% cotransducible with *ilv* (Wanner, 1983), this shows that the constitutive expression of alkaline phosphatase in this segregant was due to a genetic lesion located in the *pst* region.

To verify that the deletion in the *pstC* gene carried originally on plasmid pAN346 was present on the chromosome of the segregants which had an alkaline phosphatase constitutive phenotype, chromosomal DNA was prepared from these strains (Chapter 2, Section 2.6A) and analyzed by *in situ* DNA-DNA hybridization (Silhavy *et al.*, 1984) as described in Chapter 2 (Section 2.10). A 350bp *Sau*3A restriction endonuclease-generated fragment (from plasmid pAN92) which carries the *Bst*EII restriction site was used as a probe (Fig. 5.4). The positions of the *Sau*3A restriction endonuclease sites relative to Δ*pstC345* are such that this *Sau*3A restriction fragment, when used as a probe, had about 90 nucleotides located downstream of the 3' end

of the deletion with which it could potentially hybridize. This fragment was purified (Chapter 2, Section 2.8A) and labelled by the method of primed synthesis (Chapter 2, Section 2.10A).

Chromosomal DNA, prepared from two alkaline phosphatase constitutive segregants, and from a segregant with the phosphate-repressible alkaline phosphatase phenotype, was digested with the restriction endonuclease BstEII and electrophoresed on a 0.8% (w/v) agarose gel. This gel was then prepared for hybridization as described in Chapter 2 (Section 2.10B). Two BstEII restriction endonuclease-generated fragments, of 2.7 and 3.9kb, from the chromosomal DNA of the segregant with the phosphate-repressible alkaline phosphatase phenotype, hybridized to the probe (Fig. 5.5). A 2.7kb fragment from plasmid pAN36, which is known to carry the pst region (Cox et al., 1981), also hybridized to the probe, while no hybridization was detected with the chromosomal DNA prepared from strain DL538 (Δ[tna-glmS]), which has the pst region deleted from its chromosome. In the chromosomal DNA prepared from the two alkaline phosphatase constitutive segregants a 6.3kb BstEII restriction endonuclease-generated fragment hybridized to the probe (Fig. 5.5). This result was also obtained when the chromosomal DNA from alkaline phosphatase constitutive strains prepared by transduction of strain AN346 with bacteriophage P1 lysates of the alkaline phosphatase constitutive segregants was probed as described above (result not shown). Since the construction of the deletion ΔpstC345 involved the loss of a BstEII restriction endonuclease recognition sequence the observation that only one BstEII restriction endonuclease-

Fig. 5.5. Hybridization of DNA fragments *in situ* in an agarose gel. Chromosomal DNA (3μg) or plasmid DNA (0.3μg) was digested with the restriction endonuclease *Bst*EII. DNA fragments were separated by electrophoresis on a 0.8% (w/v) agarose gel. Following denaturation of the electrophoresed DNA and drying, the gel was hybridized *in situ* with a 350 base pair *Sau*3A restriction endonuclease-generated fragment (see Fig. 5.4) that had been labelled by primed synthesis. After washing the gel was exposed to X-ray film with an intensifying screen. (A) Plasmid pAN36 (short exposure) and (F) plasmid pAN36 (long exposure); (B), (C) and (D) chromosomal DNA from chloramphenicol-sensitive segregants of strain AN2351, with the Pi-repressible (B) and the constitutive (C,D) alkaline phosphatase phenotypes; (E) chromosomal DNA from strain DL538 (Δ[*tna*-*glmS*]). λ DNA digested with the restriction endonuclease *Hind*III and end-labelled with [α-^{32}P]dATP was used to provide molecular weight markers (not shown). The numbers represent kilobase pairs (kb). The 12kb fragment from plasmid pAN36 which hybridized to the probe includes vector (pACYC184) DNA, and is not seen in the hybridization pattern of the chromosomal DNA digestions.


A B C D E F
23.1
9.4
6.6
4.4
2.3
2.0
23.1
9.4
6.6
4.4
2.3
2.0

generated fragment, from the chromosomal DNA of the alkaline phosphatase constitutive strains, hybridizes to the probe is consistent with the incorporation of this deletion into the chromosome, near the ilv locus.

Strain AN2537 (ΔpstC345 argH pyrE entA), derived by transduction of the deletion ΔpstC345 into strain AN346 (ilvC argH pyrE entA) was chosen for further characterization, and a recA derivative (AN2538) was prepared. It was necessary to show that the deletion ΔpstC345 did not exert a polar effect on the transcription of the genes distal to the deletion. In the absence of transcriptional polarity, an alteration of phenotype caused by the presence of the deletion ΔpstC345 could be attributed solely to the mutated pstC gene. The complementation behaviour of the deletion ΔpstC345 with mutations affecting the Pst system and including the alleles pstA34, pstB401 and ΔphoU1, was analysed. The mutant alleles pstA34 and pstB401, which had previously been transferred to F-plasmids (Cox et al., 1981; G. Cox, personal communication), were transferred into strain AN2538 by conjugation (Chapter 2, Section 2.9A). The deletion ΔphoU1 was present on plasmid pAN357 (see Chapter 6), a derivative of the unit-copy plasmid vector pMF3 (Manis and Kline, 1977). Plasmid pAN357 was transferred into strain AN2538 by transformation. The partial diploids thus prepared had the deletion ΔpstC345 on the chromosome, and either the pstA34, pstB401 or ΔphoU1 allele on a plasmid. Following growth on selective media containing high phosphate, the partial diploids were found to have the phosphate-repressible alkaline phosphatase phenotype (Table 5.1) as assayed by the spray method (Chapter 2,

Table 5.1. Alkaline phosphatase phenotype of haploid (A) and partial diploid strains (B).

Partial diploid strains carrying *pst* alleles were constructed from suitable haploid strains as described in Chapter 2 (Section 2.9A). Complementation between the *pst* alleles in the partial diploid strains following growth on selective media containing high phosphate (Chapter 2, Section 2.2D) was determined by testing for alkaline phosphatase (APase) activity with the spray method (Chapter 2, Section 2.4A). The alkaline phosphatase activities of the corresponding haploid strains were also determined in the same manner. The presence and absence of alkaline phosphatase activity was equated with the constitutive and the Pi-repressible phenotypes, respectively.

Strains	APase phenotype[1]
A. Haploids (mutant allele on chromosome)	
AN2539 (wild-type)	R
AN2538 (Δ*pstC345*)	C
AN1403 (*pstB401*)	C
AN1700 (*pstA34*)	C
B. Partial diploids[2] (allele on chromosome/allele on plasmid)	
AN2564 (Δ*pstC345*/*pstA34*)	R
AN2565 (Δ*pstC345*/*pstB401*)	R
AN2566 (Δ*pstC345*/Δ*phoU1*)[3]	R
AN2568 (*pstA34*/*pstA34*)	C
AN1416 (*pstB401*/*pstB401*)	C

... /contd. ...

Table 5.1. (/contd.)

[1] R = repressed; C = constitutive.

[2] The recipient strains were AN2538 (ΔpstC345), AN1700 (pstA34) and AN1403 (pstB401), and the donor strains for the alleles pstA34 and pstB401 were AN1706 and AN1237, respectively.

[3] The mutation ΔphoU1 has not been characterized in the haploid state, but is presumed to lead to constitutive alkaline phosphatase activity because this mutation is unable to complement the phoU35 allele (see Chapter 6).

Section 2.4A), indicating that complementation between the alleles had occurred. The deletion ΔpstC345 therefore does not exert detectable polar effects on the pstA, pstB and phoU genes.

Strain AN2537 (ΔpstC345) and its isogenic sibling AN2539 (wild-type) displayed ^{32}Pi uptake rates, determined as described in Chapter 2 (Section 2.4B), of 0.5 and 33.2nmol Pi/min per mg dry weight, respectively. The periplasmic-cytoplasmic fraction was derived from both strains AN2537 and AN2539 grown on the high Pi minimal medium as described in Chapter 2 (Section 2.3C). The alkaline phosphatase activities in the fractions from both strains were assayed (Chapter 2, Section 2.4A), and found to be 1.41 (AN2537) and <0.1 (AN2539) μmol ρ-nitrophenol formed/min per mg protein.

5.4 DISCUSSION

The pstC gene has been shown to be essential for the high-affinity transport of Pi, and for the repression of alkaline phosphatase synthesis under conditions of high Pi concentration. Amemura et al. (1985) confirmed the role of the pstC gene in the regulation of alkaline phosphatase synthesis. The deletion ΔpstC345, which does not disrupt the reading frame of the pstC gene, did not exert detectable polar effects on the expression of the other pst genes located downstream of the deletion. Therefore the phenotypic effects of this deletion can be attributed to the loss of the function of the PstC protein.

The Pst system contains two hydrophobic proteins, PstC and PstA, which are essential for the transport and regulatory functions of this system. It has been suggested that the hydrophobic proteins, HisQ and HisM, of the histidine permease of S. typhimurium (Higgins et al., 1982b), and MalF and MalG, of the maltose transport system of E. coli (Treptow and Shuman, 1985), form a binding site for the transport of the substrates of these systems across the inner membrane. The hydrophobic nature of the PstC and the PstA proteins suggests that substantial portions of these proteins are embedded in the inner membrane and could be involved in the transport of Pi across this membrane. The different topologies of the PstC and the PstA proteins within the inner membrane (see Chapter 4, Section 4.5) suggests that the contribution of each protein to this process would differ. Presumably the PstA protein, an integral membrane protein, would be involved directly in the translocation of Pi across the lipid bilayer, while the PstC protein, a peripheral membrane protein, would facilitate those steps of the translocation of Pi which occur at or near the interface between the membrane and the periplasm or cytoplasm.

CHAPTER 6

ROLE OF THE phoU GENE PRODUCT IN THE PST SYSTEM

6.1 INTRODUCTION

The phoS, pstC, pstA (= phoT) and pstB genes have been shown to be essential for the transport of Pi through the Pst system, and for the repression, by high levels of extracellular Pi, of the synthesis of alkaline phosphatase (Chapter 1, Sections 1.4A and 1.4C1; Chapter 5). Zuckier and Torriani (1981) noted that one of the phoT mutants (phoT35), which was subsequently designated phoU (Amemura et al., 1982), differed from the others in that it was capable of utilizing Pi in a pit background. It was confirmed in Chapter 4 that the phoU35 mutation did not abolish Pi uptake through the Pst system. This uptake did not require a prior period of Pi starvation: it was constitutive. It therefore appeared that the PhoU protein was involved only in the regulation of the pho regulon by the Pst system (see Chapter 8).

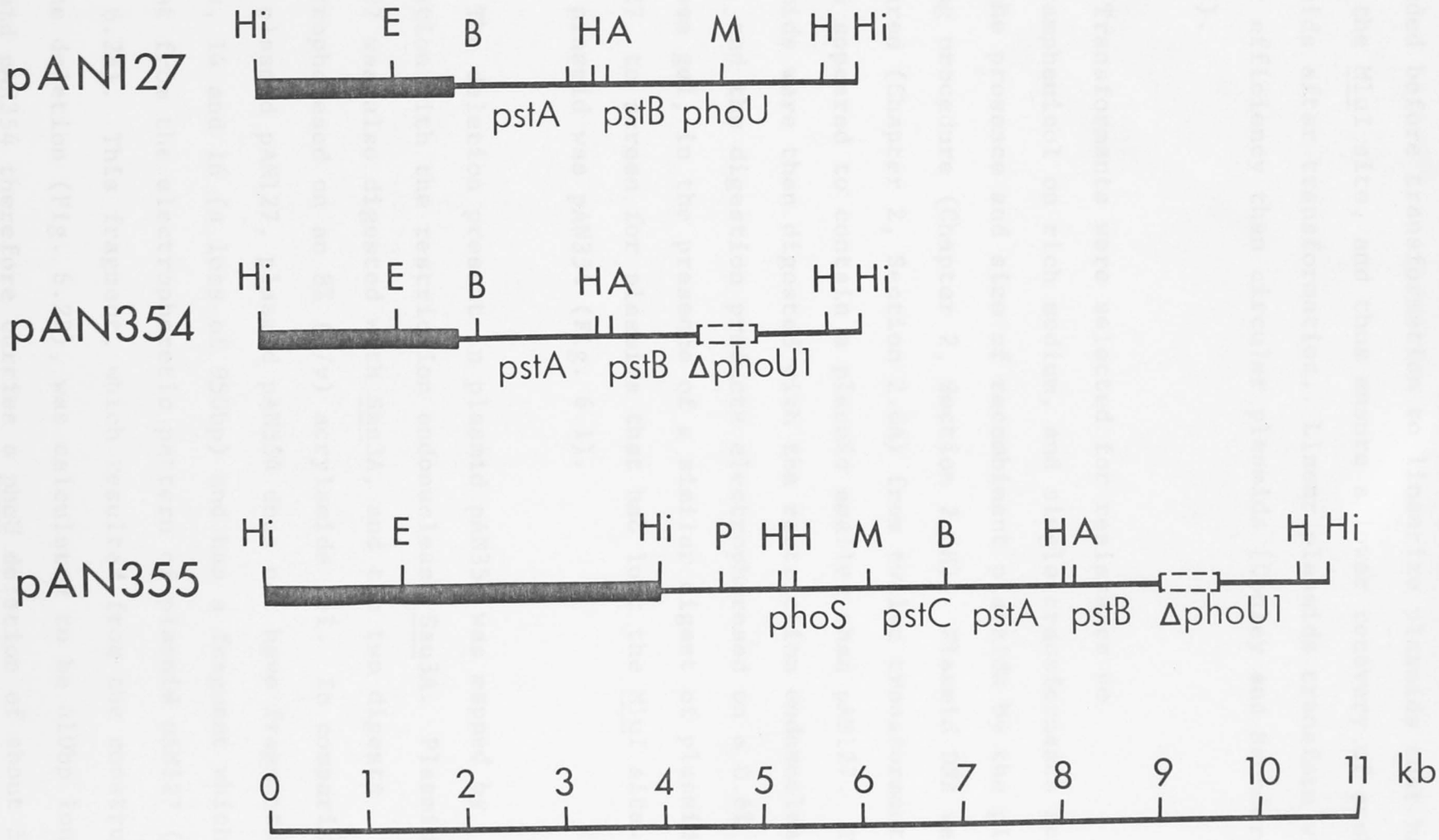
The possibility remained that the PhoU protein was involved in both Pi transport and the regulation of the pho regulon, and that the phoU35 mutation affected only the regulatory function of the protein. In the phosphoenolpyruvate:sugar phosphotransferase system (PTS) the protein III^{glc} is involved in the transport of glucose and methyl α-glucoside, and in the regulation of the synthesis and activity of adenylate cyclase and of a number of non-PTS transport systems (see Chapter 1, Section 1.3B).

To clarify the role of the phoU gene in the Pst system, a plasmid which carried a phoU deletion and wild type copies of the other pst genes was constructed. This plasmid was transformed into a strain which had the entire pst region deleted from its chromosome, and the phenotype of the resulting transformant was established with respect to high-affinity Pi transport and alkaline phosphatase synthesis.

6.2 CONSTRUCTION OF A phoU DELETION

A site for the restriction endonuclease MluI is located within the protein-coding sequence of the phoU gene (Fig. 6.1). This site, which is unique in plasmid pAN127, was used to construct a deletion in the phoU gene. Plasmid pAN127 was digested with the restriction endonuclease MluI. The linearized plasmid was treated with the nuclease Bal31 (Chapter 2, Section 2.6D), which progressively degrades both strands of double-stranded DNA from the ends (Gray et al., 1975). The reaction with the nuclease Bal31 was quenched at different time intervals after the addition of the enzyme, and the quenched reaction mixtures were electrophoresed on a 0.6% (w/v) agarose gel. DNA fragments possessing deletions of approximately 500 base pairs (bp), which appeared to be present in the incubations quenched after 5 and 10min, were extracted from the agarose gel (Chapter 2, Section 2.6J). The DNA fragments were purified and ligated with T4-DNA ligase. The ligation mixture was incubated with the restriction endonuclease MluI, before transformation into strain AN1459 (ilvC thr leu recA). (The digestion with MluI was

Fig. 6.1. Physical maps of plasmids pAN127, pAN354 and pAN355. Restriction endonuclease sites: A, AvaI; B, BstEII; E, EcoRI; H, HpaI; Hi, HindIII; M, MluI; P, PstI. Plasmid pAN354 was derived from plasmid pAN127 by digestion with the restriction endonuclease MluI, followed by treatment with the nuclease Bal31, and re-ligation. Plasmid pAN355 was constructed from plasmid pAN92 by replacement of the 5.2kb BstEII-EcoRI fragment of this plasmid with the 4.7kb BstEII-EcoRI fragment from plasmid pAN354. The physical map of plasmid pAN92 is the same as that for plasmid pAN355, with the exception that it carries a wild type phoU gene. The approximate positions of genes on the various plasmids are as shown. Plasmids pAN354 and pAN355 carry ΔphoU1, a deletion of approximately 540 nucleotides located within the phoU gene. The broken box represents the deletion, while the heavy line indicates the vector (plasmid pACYC184) portion of the plasmids. kb, kilobase pair.


pAN127
Hi E B HA M H Hi
pstA pstB phoU
pAN354
Hi E B HA H Hi
pstA pstB ΔphoU1
pAN355
Hi E Hi P HH M B HA H Hi
phoS pstC pstA pstB ΔphoU1
0 1 2 3 4 5 6 7 8 9 10 11 kb

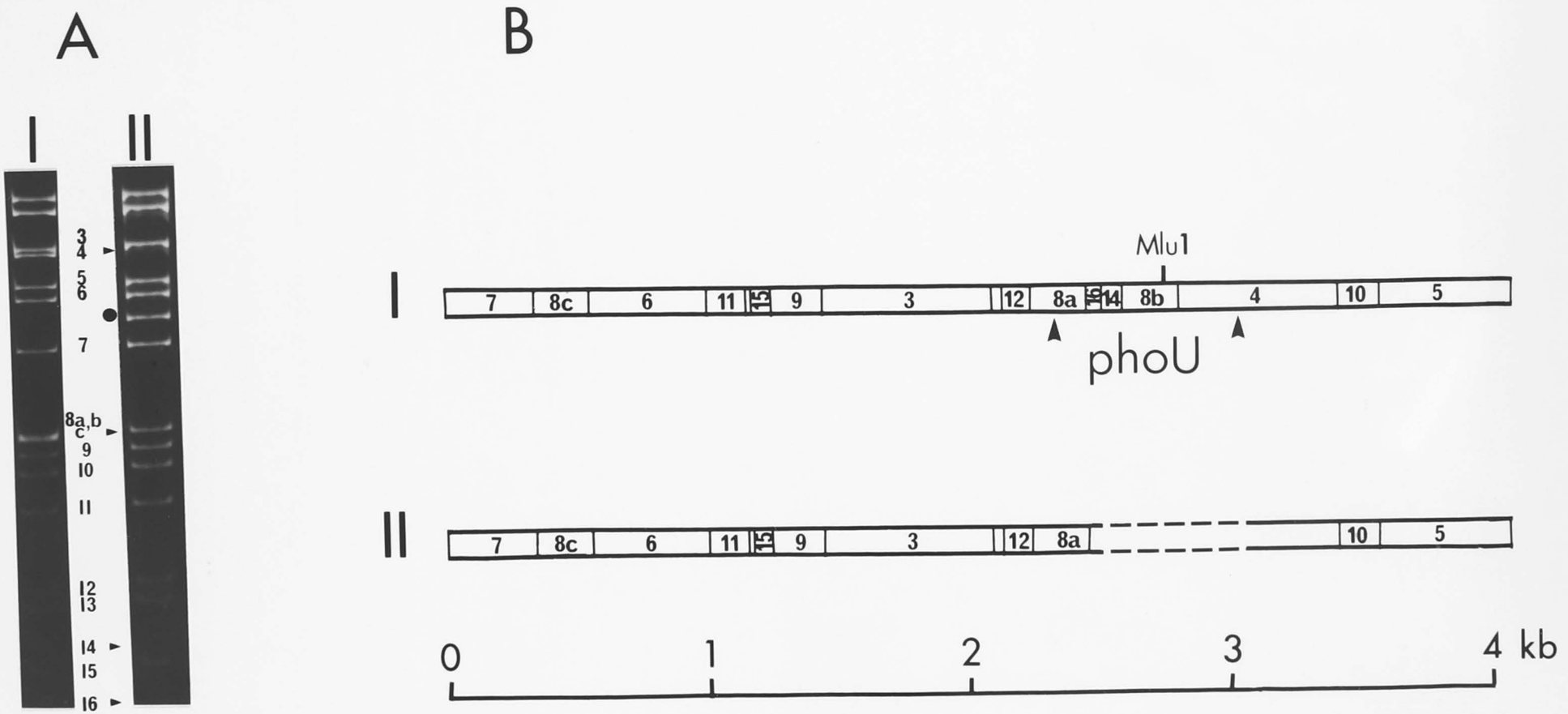
included before transformation to linearize plasmids that had not lost the MluI site, and thus ensure a lower recovery of such plasmids after transformation. Linear plasmids transform with a lower efficiency than circular plasmids [Conley and Saunders, 1984]).

Transformants were selected for resistance to chloramphenicol on rich medium, and single transformants screened for the presence and size of recombinant plasmids by the plasmid sizing procedure (Chapter 2, Section 2.6H). Plasmid DNA was prepared (Chapter 2, Section 2.6A) from twelve transformants which appeared to contain a plasmid smaller than pAN127. These plasmids were then digested with the restriction endonuclease MluI, and the digestion products electrophoresed on a 0.6% (w/v) agarose gel, in the presence of a similar digest of plasmid pAN127, to screen for plasmids that had lost the MluI site. One such plasmid was pAN354 (Fig. 6.1).

The deletion present in plasmid pAN354 was mapped by digestion with the restriction endonuclease Sau3A. Plasmid pAN127 was also digested with Sau3A, and the two digests electrophoresed on an 8% (w/v) acrylamide gel. In comparison with plasmid pAN127, plasmid pAN354 does not have fragments No. 4, 8a, 14 and 16 (a loss of 950bp) and has a fragment which is absent from the electrophoretic pattern of plasmid pAN127 (see Fig. 6.2A). This fragment, which resulted from the construction of the deletion (Fig. 6.2B), was calculated to be 410bp long. Plasmid pAN354 therefore carries a phoU deletion of about 540 nucleotides, and most of the remaining 180 nucleotides are

Fig. 6.2. A. Polyacrylamide gel electrophoresis of restriction endonuclease digests of plasmid DNA. B. Fine structure map of plasmid DNA (based on data from A), adjacent to and including the deletion ΔphoU1. In A, plasmid pAN127 (I) and plasmid pAN354 (II) were digested with the restriction endonuclease Sau3A, and the digests electrophoresed on an 8% (w/v) acrylamide slab gel as described in Chapter 2 (Section 2.6I). The fragments of non-vector DNA of the plasmids are numbered. Arrows in (II) indicate the DNA fragments (Nos. 4, 8b, 14 and 16) that are absent from the electrophoretic pattern of plasmid pAN354, but present in the pattern for plasmid pAN127. The absence of fragment No. 8b from the electrophoretic pattern of plasmid pAN354 was verified by digestion with the restriction endonuclease MluI after digestion with Sau3A (not shown). The full circle indicates the fragment present only in the electrophoretic pattern of plasmid pAN354.

In B, the Sau3A restriction endonuclease fragment maps of plasmids pAN127 (I) and pAN354 (II) are shown. With the exception of fragment No. 5, which carries some vector DNA, only fragments carrying non-vector DNA are presented. In (I) the position of the MluI restriction site used in the construction of the deletion ΔphoU1 is shown in relation to the phoU gene. Arrowheads indicate the extent of the phoU gene. In (II) the deletion ΔphoU1 is indicated by the broken line. Fragments are numbered according to the convention used in A, above. kb, kilobase pair.

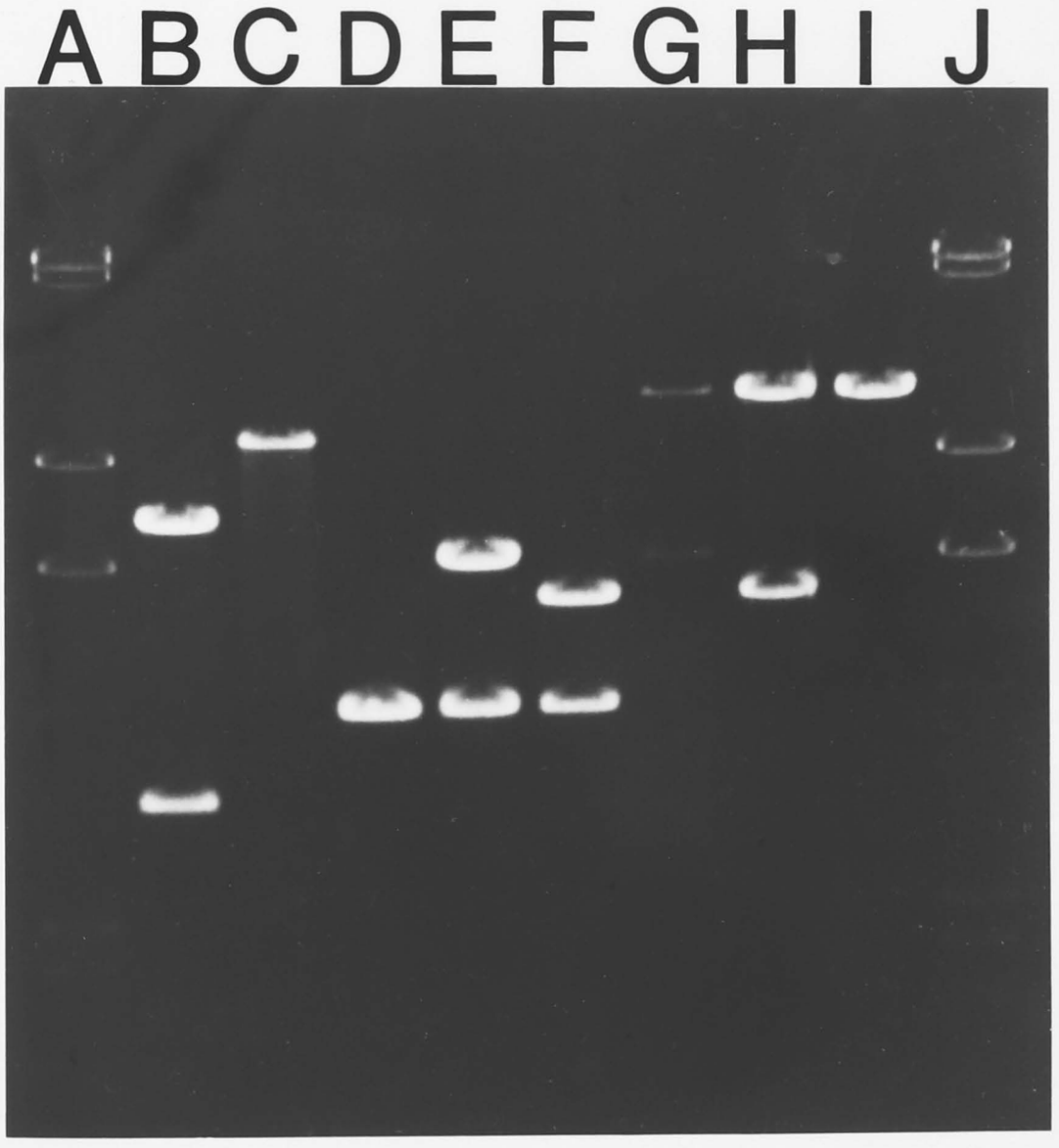

A
B
I
II
3
4
5
6
7
8a,b
c
9
10
11
12
13
14
15
16
Mlu1
7
8c
6
11
15
9
3
12
8a
16
14
8b
4
10
5
phoU
0
1
2
3
4 kb

located at the 5' end of the phoU gene (Fig. 6.2B). This deletion was designated ΔphoU1.

To facilitate the characterization of the biochemical effect of the deletion ΔphoU1 (see Section 6.3 below), plasmid pAN355 (phoS$^+$pstC$^+$A$^+$B$^+$ΔphoU1) was constructed from plasmid pAN354 (pstA$^+$B$^+$ΔphoU1) and plasmid pAN92 (phoS$^+$pstC$^+$A$^+$B$^+$phoU$^+$). Plasmids pAN92 and pAN354 were digested separately with both the restriction endonucleases BstEII and EcoRI. The digest of plasmid pAN354 was electrophoresed on a 0.6% (w/v) agarose gel, and the 4.7kb BstEII-EcoRI fragment (Fig. 6.1) was purified from the gel (Chapter 2, Section 2.6J). The digest of plasmid pAN92 was treated with calf intestinal alkaline phosphatase (Chapter 2, Section 2.6E), mixed with the 4.7kb fragment from plasmid pAN354, and the mixture ligated. The ligation mixture was transformed into strain AN1459 (ilvC thr leu recA). Transformants were selected for resistance to chloramphenicol on rich medium. Single transformants were then screened for the presence and size of recombinant plasmids by the plasmid sizing procedure (Chapter 2, Section 2.6H). Plasmid pAN355 was purified (Chapter 2, Section 2.6A) from a typical transformant adjudged to contain a plasmid smaller than plasmid pAN92.

Plasmids pAN355 and pAN92 were each digested with the restriction endonucleases MluI and HindIII, and the digests electrophoresed on a 0.6% (w/v) agarose gel. Plasmid pAN355, in comparison with plasmid pAN92, has one fewer MluI site (Fig. 6.3). The HindIII fragment containing chromosomal DNA of plasmid pAN355 is about 0.5kb shorter than the corresponding fragment of

Fig. 6.3. Agarose gel electrophoresis of plasmid DNA. Plasmid DNA was digested with the restriction endonucleases MluI or HindIII, and electrophoresed on a 0.6% (w/v) agarose gel, in the presence of λ DNA digested with HindIII as molecular weight marker. Shown are: MluI digests of pAN92 (B) and pAN355 (C); HindIII digests of λ DNA (A) and (J), pACYC184 (D), pAN92 (E), pAN355 (F), pAN356 (G), pAN357 (H) and pMF3 (I).


A B C D E F G H I J

plasmid pAN92 (Fig. 6.3), verifying the presence of the deletion Δ*phoU1* on the former plasmid.

6.3 PRELIMINARY ANALYSIS OF Δ*phoU1*

The deletion Δ*phoU1*, unlike the deletion Δ*pstC345* (see Chapter 5), could not be transferred to the chromosome of strain AN2351 (*polA12*^{ts}). All chloramphenicol-sensitive segregants obtained by transformation of AN2351 with either plasmid pAN354 or plasmid pAN355 had the Pi-repressible alkaline phosphatase phenotype. (The deletion Δ*phoU1* could not complement the *phoU35* mutation [see Table 6.1 below], and was therefore expected, like the *phoU35* allele, to lead to constitutive expression of alkaline phosphatase).

The biochemical effect of the deletion Δ*phoU1* was then investigated in strain HR299, a *recA* derivative of strain DL538 (Δ[*tna-glmS*]) which has the *pst* region deleted from its chromosome. No chloramphenicol-resistant transformants were obtained when plasmid pAN355 (*phoS*$^+$*pstC*$^+$*A*$^+$*B*$^+$Δ*phoU1*) was transformed into strain HR299. Since plasmid pAN92 (*phoS*$^+$*pstC*$^+$*A*$^+$*B*$^+$*phoU*$^+$) could successfully transform this strain, it seemed likely that the failure to obtain transformants with the multicopy plasmid pAN355 was due to either the presence of the deletion Δ*phoU1* *per se*, or to the presence of multiple copies of this deletion. To check this a unit-copy plasmid carrying the *phoU* deletion was required.

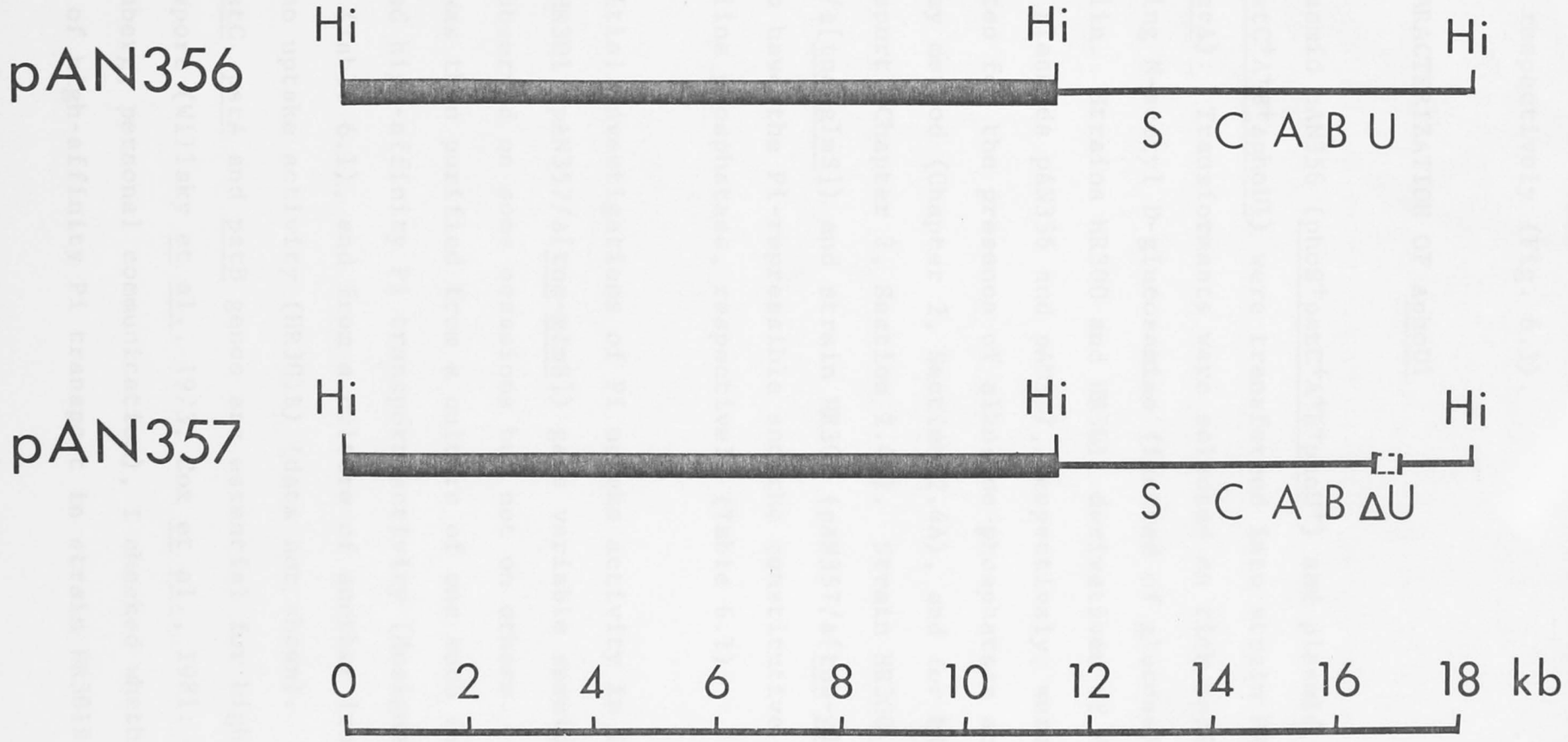
6.4 CONSTRUCTION OF PLASMID pAN357

Plasmid pAN355 and the unit-copy plasmid vector pMF3 (Manis and Kline, 1977) were digested separately with the restriction endonuclease HindIII. The digest of plasmid pAN355 was electrophoresed on a 0.6% (w/v) agarose gel, and the 6.2kb HindIII fragment (see Fig. 6.1) was purified as described in Chapter 2 (Section 2.6J). The digest of plasmid pMF3 was treated with calf intestinal alkaline phosphatase (Chapter 2, Section 2.6E), and mixed with the purified 6.2kb HindIII fragment from plasmid pAN355. The mixture was ligated, and transformed into strain AN1459 (ilvC thr leu recA). Transformants were selected for resistance to ampicillin on rich medium. Single transformants were then screened for the presence and size of recombinant plasmids, by the plasmid sizing procedure for plasmids constructed with the unit-copy plasmid pMF3 as vector (Chapter 2, Section 2.6H). Plasmid DNA was purified, from strains which contained a plasmid larger than pMF3, by a Triton-lysis procedure and ultracentrifugation on a caesium chloride gradient (Chapter 2, Section 2.6A). One such plasmid is pAN357 (Fig. 6.4).

Plasmid pAN356 (Fig. 6.4), which carried the 6.7kb HindIII fragment of plasmid pAN92 (see Fig. 6.1), was constructed in the same way as plasmid pAN357 (see above), with plasmid pMF3 as the vector.

Plasmids pAN356 and pAN357, as judged by digestion with the restriction endonuclease HindIII and electrophoresis of the reaction products on a 0.6% (w/v) agarose gel, clearly have the

Fig. 6.4. Physical maps of plasmids pAN356 and pAN357. Abbreviations: A, pstA; B, pstB; C, pstC; S, phoS; U, phoU; ΔU, ΔphoU1; Hi, HindIII; kb, kilobase pair. The approximate locations of the pst genes on the plasmids are shown. The sites on the plasmids for the restriction endonuclease HindIII are indicated. The deletion ΔphoU1 is indicated by the broken box, and the vector (plasmid pMF3) portion of the plasmids is represented by the heavy line.


pAN356
Hi
Hi
Hi
S C A B U
pAN357
Hi
Hi
Hi
S C A B ΔU
0 2 4 6 8 10 12 14 16 18 kb

6.7kb and the 6.2kb HindIII fragments from plasmids pAN92 and pAN355, respectively (Fig. 6.3).

6.5 CHARACTERIZATION OF ΔphoU1

Plasmid pAN356 (phoS$^+$pstC$^+$A$^+$B$^+$phoU$^+$) and plasmid pAN357 (phoS$^+$pstC$^+$A$^+$B$^+$ΔphoU1) were transformed into strain HR299 (Δ[tna-glmS] recA). Transformants were selected on rich medium containing N-acetyl D-glucosamine (instead of glucose) and ampicillin. Strains HR300 and HR301, derivatives of HR299 which carried plasmids pAN356 and pAN357, respectively, were purified and tested for the presence of alkaline phosphatase activity by the spray method (Chapter 2, Section 2.4A), and for high-affinity Pi transport (Chapter 2, Section 2.4B). Strain HR300 (pAN356/Δ[tna-glmS]) and strain HR301 (pAN357/Δ[tna-glmS]) were found to have the Pi-repressible and the constitutive phenotypes of alkaline phosphatase, respectively (Table 6.1).

Initial investigations of Pi uptake activity in isolates of strain HR301 (pAN357/Δ[tna-glmS]) gave variable results, with Pi uptake observed on some occasions but not on others. Plasmid pAN357 was then purified from a culture of one such isolate which had high-affinity Pi transport activity (designated HR301A) (Table 6.1), and from a culture of another isolate which showed no uptake activity (HR301B) (data not shown). Since the phoS, pstC, pstA and pstB genes are essential for high-affinity Pi transport (Willsky et al., 1973; Cox et al., 1981; Chapter 5; H. Rosenberg, personal communication), I checked whether the absence of high-affinity Pi transport in strain HR301B was due to

Table 6.1. Alkaline phosphatase phenotype and phosphate uptake rates of various strains of *E. coli*.

The alkaline phosphatase (APase) activity of cells grown on rich medium, containing excess (approx. 4mM) phosphate, was determined by the spray method (Chapter 2, Section 2.4A). The presence and absence of alkaline phosphatase activity was equated with the constitutive and the Pi-repressible phenotypes, respectively. Phosphate (Pi) uptake rates were measured as described in Chapter 2 (Section 2.4B).

Strain	APase phenotype[a]	Pi uptake[b]
HR299 (Δ[*tna*-*glmS*])	C	1.0
HR300 (pAN356/Δ[*tna*-*glmS*])	R	42.9
HR301A (pAN357/Δ[*tna*-*glmS*])	C	24.2[c]
HR302 (pAN357/*phoU35*)	C	ND

[a] C = constitutive; R = repressed.

[b] Pi uptake rates are expressed as nmol Pi/min per mg dry wt.

[c] This represents the rate of Pi uptake after a 2h period of Pi starvation, and is 25-50% higher than the rate measured prior to starvation.

ND, Not determined.

mutations in the genes comprising the pst region of plasmid pAN357 other than ΔphoU1. The ability of the two preparations of plasmid pAN357 to complement mutations affecting the Pst system was then tested (Chapter 2, Section 2.9D), using as the criterion the restoration of the Pi-repressible phenotype of alkaline phosphatase, which was tested by the spray method (Chapter 2, Section 2.4A). When the transforming plasmid pAN357 was prepared from strain HR301A, complementation was found in all transformants carrying the phoS64, ΔpstC345, and pstB401 mutations, and 98% of those with the pstA2 mutation on the chromosome (Table 6.2). (The original preparation of plasmid pAN357, obtained from the pst^+ strain AN1459, showed full complementation of the four alleles [data not shown]).

Plasmid pAN357, purified from strain HR301B, showed a very low level of complementation (4%) of the phoS64 allele, and varying levels of complementation of the other mutant alleles (Table 6.2). The absence of Pi transport in this strain is thus due to a genetic lesion (s), other than ΔphoU1, on the plasmid. These lesions arose presumably because "authentic" plasmid pAN357 was detrimental to the growth of strains such as HR299, which carry a total deletion of the pst region on the chromosome.

Strain HR301A, carrying "authentic" plasmid pAN357 ($phoS^+pstC^+A^+B^+$ΔphoU1), had a Pi uptake rate half that of strain HR300, carrying plasmid pAN356 ($phoS^+pstC^+A^+B^+phoU^+$) (Table 6.1). In comparison, the parent strain HR299 (Δ[tna-glmS]) had a Pi uptake rate less than one-fortieth of that of strain HR300. The Pi uptake seen in strain HR301A is constitutive: it does not

Table 6.2. Genetic complementation tests of *pst* mutant alleles.

recA derivatives of strains carrying the *pst* mutations indicated below were transformed with plasmid pAN357, which was derived from either strain HR301A or strain HR301B. Chloramphenicol-resistant transformants were selected and tested for alkaline phosphatase activity by the spray method (Chapter 2, Section 2.4A) following growth on rich medium, which contains excess (approx. 4mM) phosphate. The absence of alkaline phosphatase (APase) activity was equated with the Pi-repressible phenotype. The proportion of colonies with this phenotype is shown below.

Transforming plasmid	Mutant allele (on chromosome)			
	phoS64	Δ*pstC345*	*pstA2*	*pstB401*
	per cent APase-negative colonies (repressed)			
pAN357 ex-HR301A	100	100	98	100
pAN357 ex-HR301B	4	100	95	68

require a prior period of Pi starvation (Table 6.1).

6.6 DISCUSSION

It was shown that the phoU gene is not essential for high-affinity Pi transport. Strain HR301, carrying a total deletion of the pst region on the chromosome, and a plasmid with a phoU deletion, had a Pi uptake rate of 24.2 nmol/min per mg dry wt. In comparison, strains carrying mutations in the phoS, pstC, pstA and pstB genes have a Pi uptake rate of 0.5-1.5 nmol/min per mg dry wt (Chapters 4 and 5; H. Rosenberg, personal communication). Clearly the role, if any, of the phoU gene in the Pi transport process differs from that of the other pst genes. However the phoU gene product may be required for maximal rates of uptake through the Pst system (see Table 6.1). A role for the phoU gene in the Pst system is postulated in Chapter 8.

It is not likely that the lower uptake rate of strain HR301A, compared to that of strain HR300 (Table 6.1), was caused by a substantial portion of the cells of this strain having plasmids with mutations other than ΔphoU1. Plasmid pAN357, prepared from the cells immediately after Pi transport assays, showed nearly full complementation in tests with strains carrying mutations in the phoS, pstC, pstA and pstB genes (see Table 6.2).

The deletion Δ(tna-glmS) removes about 17kb of DNA, including the pst region, from the chromosome. Plasmid pAN356 carries about 7kb of chromosomal DNA, comprising genes which have been identified in Chapter 4 (Fig. 4.7). The ability of this

plasmid to restore high-affinity Pi transport and the Pi-repressible alkaline phosphatase phenotype in the strain carrying the deletion Δ(tna-glmS) confirms that within the 17kb region of the chromosome all the genes comprising the Pst system have been identified.

Plasmid pAN357, prepared from strain HR301B, carried mutations other than ΔphoU1 located mainly in the phoS and the pstB genes (Table 6.2). Since all the pst genes are similar in size, this observation suggests that the phoS and the pstB genes may carry mutational "hot-spots". Another possible explanation is that large portions of the PBP and the PstB protein are essential to the Pi transport process and the regulation of alkaline phosphatase synthesis, and this would decrease the number of phenotypically silent mutations in these genes. It is known that periplasmic binding proteins have several domains which are essential for substrate transport (see Hengge and Boos, 1983). Passage of plasmid pAN357 through strain HR299 (Δ[tna-glmS]) is a good method for isolating mutations in the pst genes, particularly in phoS and pstB. This method has the advantage that the mutations are generated on a plasmid, enabling easy characterization.

CHAPTER 7

PURIFICATION OF THE PhoU PROTEIN

7.1 INTRODUCTION

It was shown in the previous chapter that the phoU gene is not essential for high-affinity Pi transport through the Pst system. The PhoU protein probably mediates the regulation by the Pst system of gene expression in the pho regulon (see Chapter 8). As part of a study to elucidate the mechanism by which the PhoU protein regulates gene expression, a procedure was developed for the rapid purification of this protein in high yield. Amino acid analysis of the purified protein would confirm the translational start-site and the reading frame proposed for the phoU gene in Chapter 3. In future studies the purified protein could be tested in vitro for DNA-binding activity, in an attempt to assign a repressor or activator role for this protein.

The purification of proteins has been facilitated by the use of plasmids specially constructed to over-produce the products of genes cloned into such plasmids. One way of achieving this is by increasing the frequency of transcription of a gene by cloning it into the right orientation with respect to a strong promoter. The resulting increase in the level of mRNA should lead, if the protein synthesizing machinery can cope with the increased demand for protein synthesis, to a higher level of the corresponding protein. Such promoters can usually be "switched" on or off in a simple manner, e.g. by thermal or chemical means.

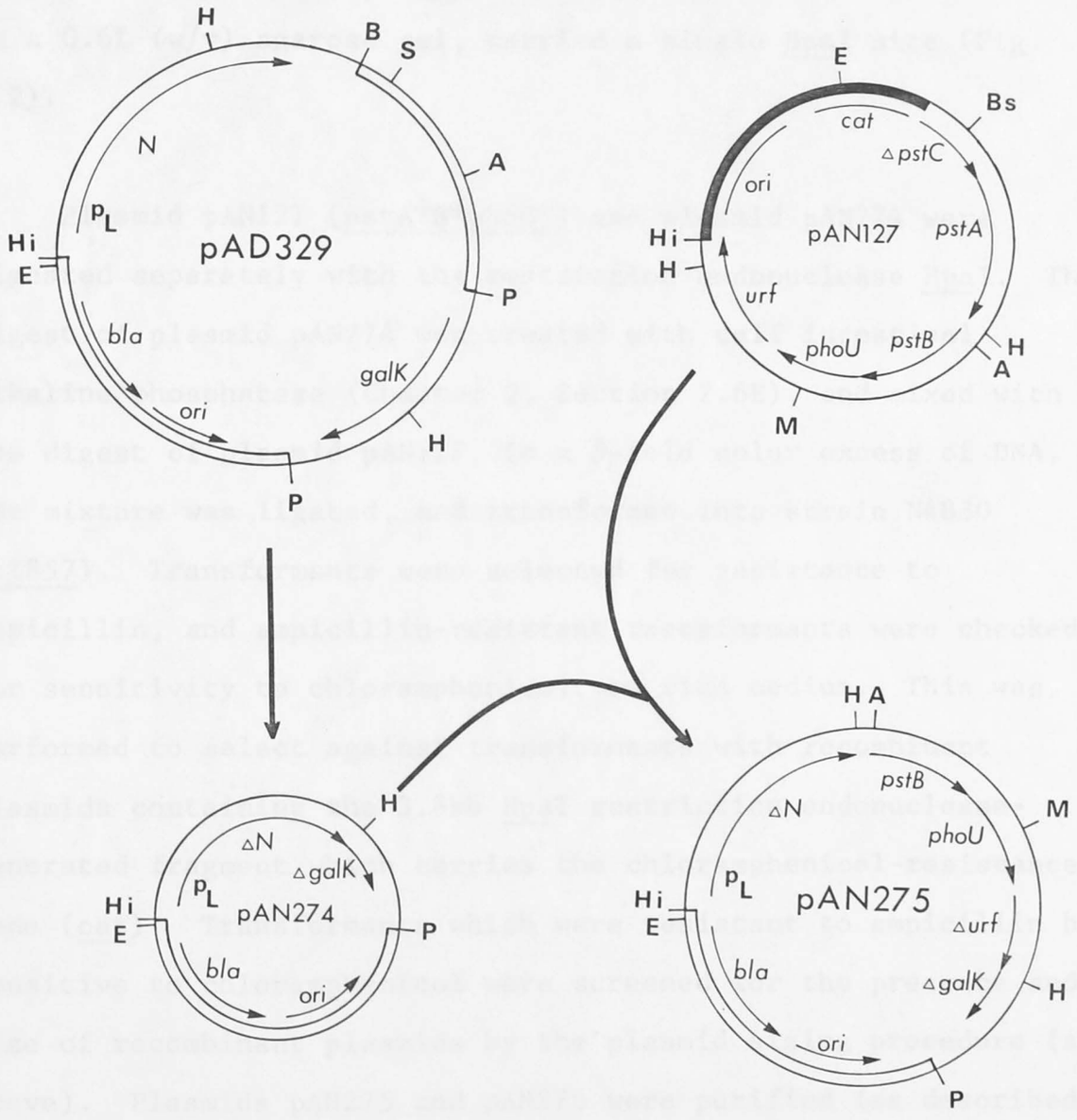
To over-produce the PhoU protein, the *phoU* gene was cloned into a plasmid with the bacteriophage λ promoter p_L, which is subject to repression by the λ *cI* protein (see Rosenberg *et al.*, 1983). This plasmid and its derivatives were always maintained in a strain with the *cI857* gene on the chromosome. This allele produces a temperature-sensitive *cI* repressor. At 30°C this repressor is fully functional, while at higher temperatures it is progressively inactivated, resulting in a greatly increased transcription of genes cloned in the right orientation with respect to the promoter p_L, and thus over-production of the corresponding gene product.

7.2 SUB-CLONING OF THE *phoU* GENE INTO A PLASMID CARRYING p_L

The *pstB* and the *phoU* genes are present on the 2.3kb *Hpa*I restriction endonuclease-generated fragment of plasmid pAN127 (Fig. 7.1). This fragment was sub-cloned into the *Hpa*I restriction site located downstream of the promoter p_L in plasmid pAN274, which was constructed from plasmid pAD329 (Fig. 7.1).

Plasmid pAD329 (gift from N.E. Dixon) was digested with the restriction endonuclease *Hpa*I, and re-ligated. The ligation mixture was transformed into strain N4830 (*cI857*) (Gottesman *et al.*, 1980) and transformants were selected for resistance to ampicillin on rich medium after growth at 30°C. (This strain and its plasmid-bearing derivatives were always grown at 30°C, unless otherwise stated). Single transformants were screened for the presence and size of recombinant plasmids by the plasmid sizing procedure (Chapter 2, Section 2.6H). Plasmid pAN274 was

Fig. 7.1. Physical maps and derivation of various plasmids. Restriction endonuclease sites: A, AvaI; B, BamHI; Bs, BstEII; E, EcoRI; H, HpaI; Hi, HindIII; M, MluI; P, PvuII; S, SalI. Plasmid pAN274 (4.7kb) was derived from plasmid pAD329 (8.3kb) by deletion of the 3.6kb HpaI restriction endonuclease-generated fragment. Plasmid pAN275 (7.0kb) was constructed by sub-cloning of the 2.3kb HpaI fragment of plasmid pAN127 (6.1kb) into the HpaI site of plasmid pAN274. The physical map of another plasmid, pAN276 (not shown), is the same as that of plasmid pAN275, except that the 2.3kb HpaI fragment is in the opposite orientation to that shown for plasmid pAN275. The approximate locations of the genes encoding the Pst system, β-lactamase (bla), chloramphenicol acetyl transferase (cat) and the λ N protein (N), and the origins of replication (ori) of the plasmids, are shown. The unidentified reading frame (urf) located beyond the phoU gene is probably part of the bglC gene (Reynolds et al., 1985). Thin arrows indicate the direction of genes and transcripts. Open boxes represent DNA from the plasmid vector pBR322, and the filled box DNA from the plasmid vector pACYC184.

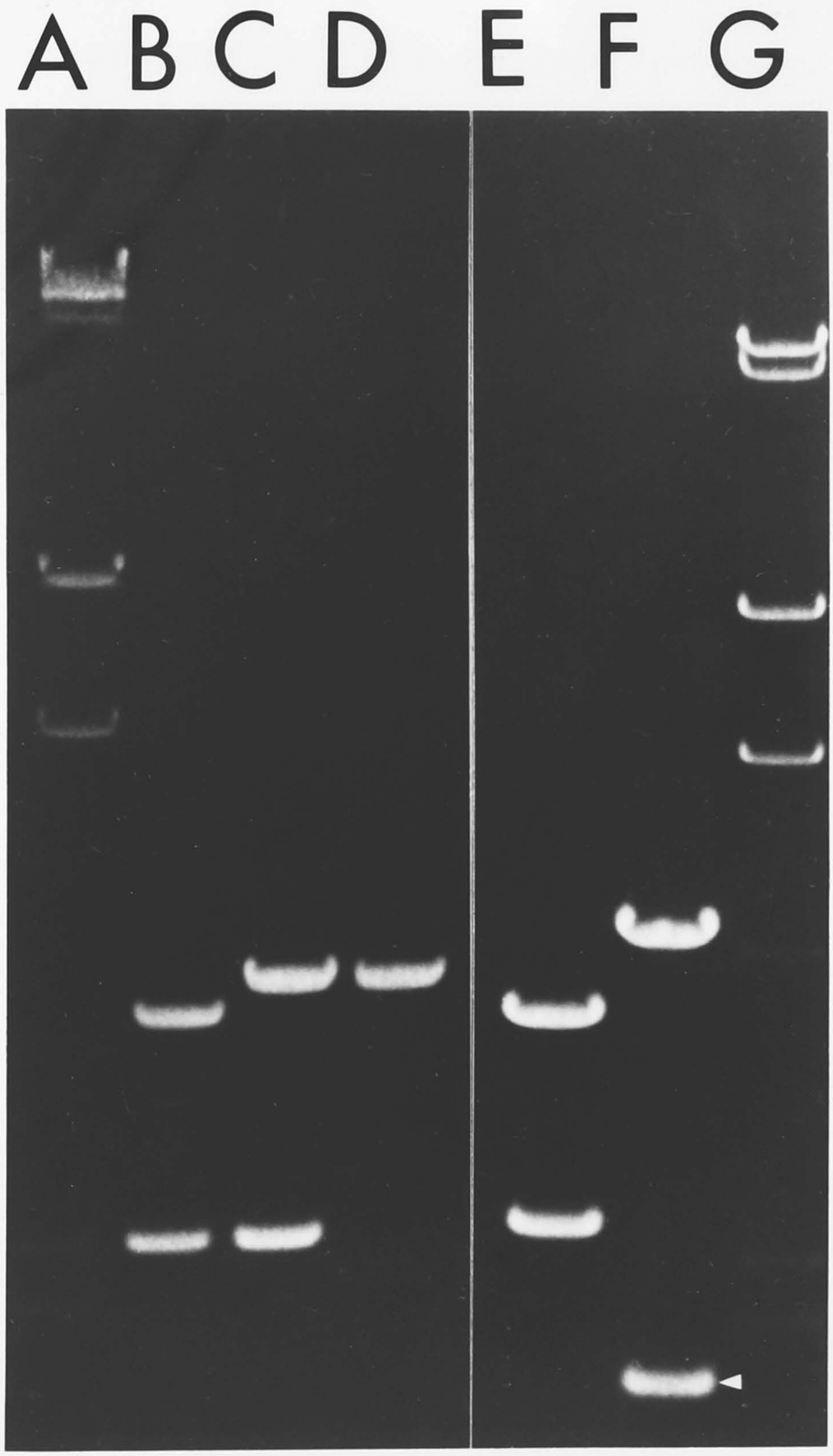

H
B
S
N
A
pAD329
P
Hi
E
p_L
bla
galK
ori
H
P
E
Bs
cat
ΔpstC
ori
pAN127
pstA
Hi
H
urf
phoU
pstB
H
A
M
H A
pstB
ΔN
M
phoU
pAN275
Hi
E
p_L
Δurf
bla
ΔgalK
H
ori
P
H
ΔN
ΔgalK
pAN274
Hi
E
p_L
P
bla
ori

purified by a Triton-lysis procedure and ultracentrifugation on a caesium chloride gradient (Chapter 2, Section 2.6A) from a typical transformant which appeared to carry a plasmid smaller than pAD329. Plasmid pAN274, as judged by digestion with the restriction endonuclease HpaI and electrophoresis of the digest on a 0.6% (w/v) agarose gel, carried a single HpaI site (Fig. 7.2).

Plasmid pAN127 (pstA$^+$B$^+$phoU$^+$) and plasmid pAN274 were digested separately with the restriction endonuclease HpaI. The digest of plasmid pAN274 was treated with calf intestinal alkaline phosphatase (Chapter 2, Section 2.6E), and mixed with the digest of plasmid pAN127, in a 5-fold molar excess of DNA. The mixture was ligated, and transformed into strain N4830 (cI857). Transformants were selected for resistance to ampicillin, and ampicillin-resistant transformants were checked for sensitivity to chloramphenicol, on rich medium. This was performed to select against transformants with recombinant plasmids containing the 3.8kb HpaI restriction endonuclease-generated fragment which carries the chloramphenicol-resistance gene (cat). Transformants which were resistant to ampicillin but sensitive to chloramphenicol were screened for the presence and size of recombinant plasmids by the plasmid sizing procedure (see above). Plasmids pAN275 and pAN276 were purified (as described above for plasmid pAN274) from typical transformants which appeared to contain a plasmid larger than pAN274.

Plasmid pAN275 and plasmid pAN276 (data not shown) were digested with the restriction endonuclease HpaI. The digests

Fig. 7.2. Agarose gel electrophoresis of plasmid DNA. Plasmid DNA was digested with the restriction endonucleases indicated below, and the digests electrophoresed on a 0.6% (w/v) agarose gel, with λ DNA digested with the restriction endonuclease HindIII as the molecular weight marker. Shown are: HindIII digests of λ DNA (A) and (G); HpaI digests of pAN127 (B), pAN275 (C), and pAN274 (D); AvaI and HindIII digests of pAN276 (E) and pAN275 (F). The 1.9kb AvaI-HindIII fragment, which is expected if the 2.3kb HpaI fragment of plasmid pAN127 has been sub-cloned into plasmid pAN274 in the right orientation with respect to the promoter p_L (see Fig. 7.1), is indicated with an arrow in F.


A B C D E F G

were electrophoresed on a 0.6% (w/v) agarose gel, together with similar digests of plasmids pAN127 and pAN274. It is clear that plasmid pAN275 carries the 2.3kb HpaI restriction endonuclease-generated fragment from plasmid pAN127. Plasmid pAN275 and plasmid pAN276 carry this 2.3kb HpaI fragment in the "right" and the "wrong" orientation, respectively, with respect to the promoter p_L. These plasmids were digested separately with the restriction endonucleases AvaI and HindIII, and the digests electrophoresed on a 0.6% (w/v) agarose gel. A 1.9kb AvaI-HindIII fragment, which is seen only if the 2.3kb HpaI fragment has been cloned in the "right" orientation (see Fig. 7.1), is present in the electrophoretic pattern for plasmid pAN275, and is not seen in the pattern for plasmid pAN276 (Fig. 7.2).

7.3 OVER-PRODUCTION OF THE PhoU PROTEIN

Plasmids pAN275 and pAN276 were transformed into strain N4830 (cI857), and transformants were selected for ampicillin resistance on rich medium. Strains HR303 (pAN275/cI857) and HR304 (pAN276/cI857) were purified on selective medium in the presence of the antibiotic. These strains were grown in 10ml of a glucose-Luria broth containing ampicillin to a cell density at 595nm of 0.6. The cultures were then transferred to another water bath and maintained at 40°C for 2h. The protein composition of the cells was then determined by gel electrophoresis following cell lysis, as described in Chapter 2 (Section 2.3D). Relatively large amounts of two proteins were identified in the electrophoretic pattern of strain HR303

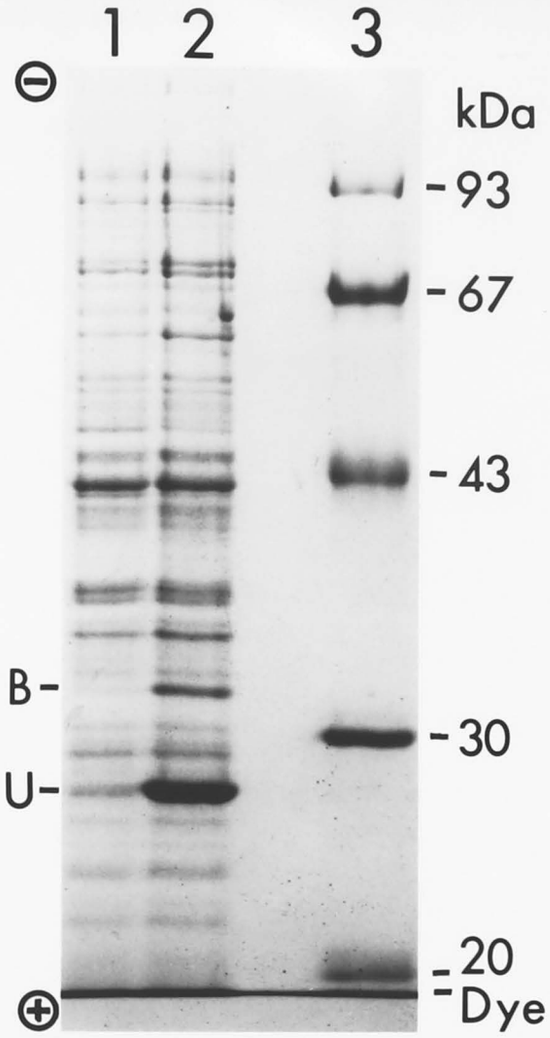
(pAN275/cI857), but not in that of strain HR304 (pAN276/cI857) (Fig. 7.3A).

Membranes were prepared from strain HR303 (grown in a 40 litre culture, see below), and subjected to two-dimensional gel electrophoresis (Chapter 2, Section 2.5C). The two-dimensional gel electrophoretogram (Fig. 7.3B) showed the presence of two proteins which were identified as the PhoU and the PstB proteins by co-electrophoresis with the corresponding ^{35}S-labelled peptides from *in vitro* transcription/translation with plasmid DNA as template (result not shown). It is evident that the PhoU protein is over-produced to a greater extent than the PstB protein (Fig. 7.3).

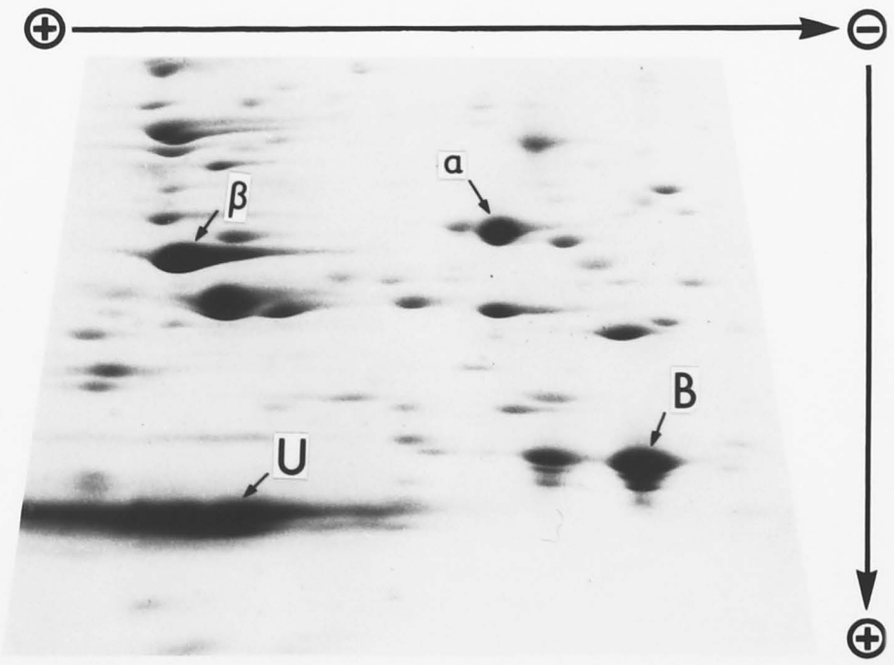
For large scale over-production of the PhoU protein, strain HR303 (pAN275/cI857) was grown in a Fermacell fermenter (New Brunswick Scientific Co., New Jersey, U.S.A.) containing 20 litres of a glucose-Luria broth supplemented with ampicillin, thymine and the appropriate growth requirements. The bulk growth medium was inoculated with one litre of an overnight culture, prepared as described in Chapter 2 (Section 2.3B). At a cell density at 595nm of 0.6, the incubation temperature of the culture was increased to 40°C. Growth was continued for 2h, and the culture (at a cell density at 595nm of 2.1) was then harvested in a Sharples centrifuge. The cells (62g) were resuspended in 32ml of a buffer containing 50mM Tris, pH7.5, 0.1mM EDTA and 2mM DTT (Buffer B). The cell suspension was then frozen in liquid nitrogen and stored at -70°C (for up to 2 months) until required.

Fig. 7.3. Polyacrylamide gel electrophoresis of proteins. A. Cells of strain HR303 (pAN275/cI857) and strain HR304 (pAN276/cI857), from 10ml cultures prepared as described in Section 7.3, were lysed (Chapter 2, Section 2.3D) and the lysates electrophoresed on a 12% (w/v) polyacrylamide gel containing 0.1% (w/v) SDS (Chapter 2, Section 2.5D). 1. Strain HR304. 2. Strain HR303. 3. Molecular weight standards: phosphorylase b [93 kilodaltons (kDa)], bovine serum albumin (67kDa), ovalbumin (43kDa), carbonic anhydrase (30kDa), and soybean trypsin inhibitor (20kDa). B. The membrane fraction was prepared as described in Chapter 2 (Section 2.3C) from strain HR303 and about 0.5mg subjected to two-dimensional gel electrophoresis (Chapter 2, Section 2.5C). Electrophoresis in the first dimension (isoelectric focusing) is in the horizontal direction, and in the second dimension (SDS-PAGE) in the vertical direction, of the figure. The short arrows refer to particular proteins identified by electrophoresis with authentic samples or with [^{35}S]methionine-labelled peptides derived from *in vitro* transcription/translation. Abbreviations: α and β, the α- and β-subunits, respectively, of the membrane-bound ATPase (Senior *et al.*, 1979); B, PstB protein; U, PhoU protein.

A



B



7.4 ISOLATION OF THE PhoU PROTEIN

Initial attempts at purifying the PhoU protein, involving differential centrifugation of cell extracts from strain HR303 (pAN275/cI857), suggested the possibility that in cells of this strain which had been induced for the synthesis of the PhoU protein, this product formed aggregates. To test this possibility, strains HR303 (pAN275/cI857) and HR304 (pAN276/cI857) were grown up in 10ml cultures, incubated at 40°C, and harvested as described in Section 7.3. The cells were then examined by electron microscopy. Structures resembling organelles were seen in the cytoplasm of strain HR303, but not in that of strain HR304 (Fig. 7.4). These organelle-like structures presumably contained the PhoU protein.

The method for the purification of the PhoU protein (see below) involved differential high-speed centrifugation of the lysate from induced cells of strain HR303 (pAN275/cI857), which contained PhoU protein in the form of an aggregate (see Fig. 7.4). The conditions at each step of the purification procedure were chosen to maintain the protein in its aggregated form.

The details of the purification (Table 7.1) were as follows: all operations were carried out at 4°C unless otherwise stated. Step I: the frozen cell suspension (12g of cell pellet in a total volume of 19ml) was thawed gradually and diluted with Buffer B (Section 7.3) to about 56ml. Additions were then made from concentrated solutions in the order stated to final concentrations shown, of the following: NaCl (250mM), DTT (2mM),

Fig. 7.4. Electron micrographs of strains HR303 (pAN275/cI857) and HR304 (pAN276/cI857). These strains were grown at 30°C and "induced" as described in Section 7.3. The fixation and post-fixation of the cells, and the electron microscopy, was performed by S.C. Tiwari (Department of Developmental Biology, Research School of Biological Sciences, Australian National University). A, B, and C: Cells of strain HR303 at a magnification of 31,000x (A), 43,000x (B) and 68,000x (C). D. Cells of strain HR304 at a magnification of 43,000x. Arrowheads indicate the positions of organelle-like structures found in the cytoplasm of strain HR303. These structures are not seen in the cytoplasm of cells of strain HR304.

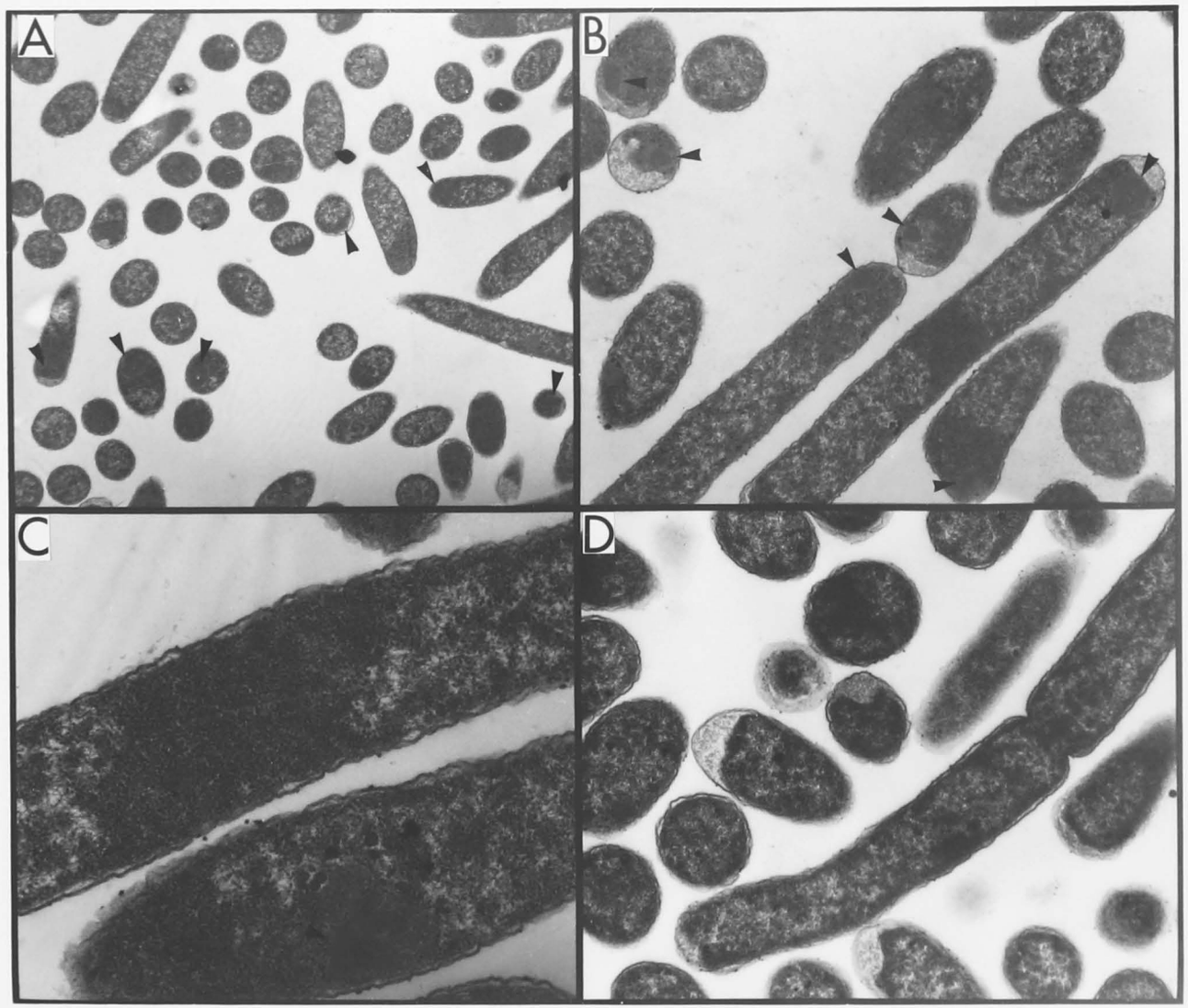

A
B
C
D

Table 7.1. Purification of PhoU protein from cells of strain HR303 (pAN275/cI857).

The PhoU protein was purified as described in Section 7.4.

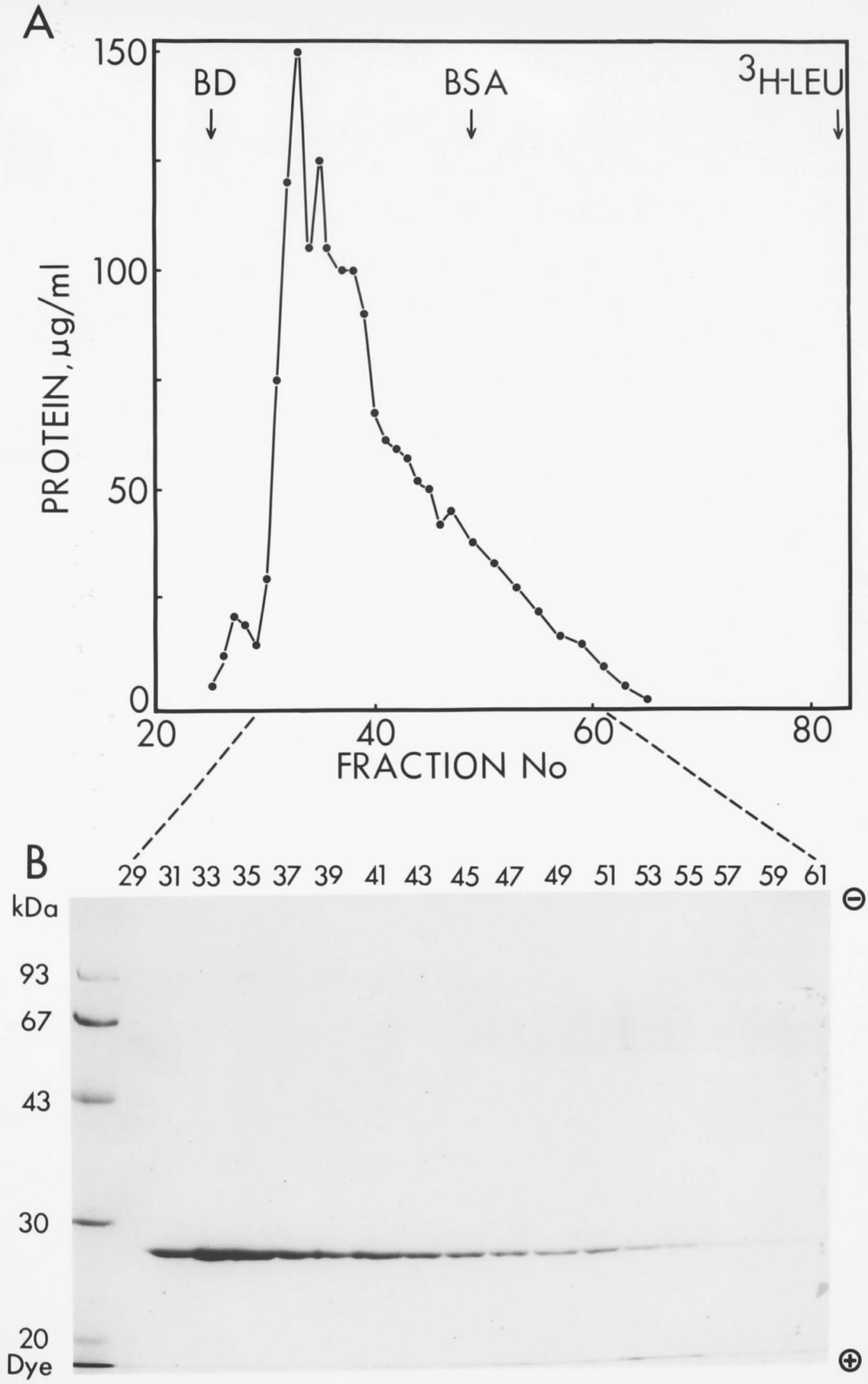
Steps	Total protein (mg)	PhoU (mg)[a]	Purification ratio	Yield (%)
I. Crude lysate	438	≈40	1	100
II. 254,000xg pellet	55.1	13.1	2.6	33
III. Triton X-100 extraction	8.4	6.1	8.0	15
IV. Ammonium sulphate fractionation	6.5	6.0	10.1	15
V. Sephadex G-200[b]	1.84	1.84	11.0	5

[a] Determined by densitometric scanning after one-dimensional SDS gel electrophoresis (see Fig. 7.6) of samples of steps I-V and integration of the trace.

[b] Fraction V was derived from 3mg of Fraction IV, and the values are adjusted accordingly.

EDTA (1mM), egg lysozyme (250µg/ml), and T4 lysozyme (≃0.1µg/ml). The suspension (60ml) was distributed into three centrifuge tubes. These were kept on ice for 15min, frozen in liquid nitrogen for 1min, and kept at room temperature for 30min. The contents were then thawed at 4°C, and centrifuged at 15000xg for 20min. The supernatant (Fraction I, 35ml) was kept. Step II: Fraction I was centrifuged at 254000xg for 2h and the pellet was resuspended in a solution containing 50mM Tris, pH7.5, 0.1mM EDTA, 2mM DTT, 100mM NaCl and 15% (w/v) glycerol (Buffer C) (Fraction II, 10ml). Step III: Triton X-100 (0.05%, w/v) was added to Fraction II. The suspension was kept on ice for 30min, agitated occasionally, and centrifuged at 40000xg for 25min. The pellet was resuspended in Buffer C to a final volume of 10ml and dialysed for 8h periods against four changes of 25 volumes of Buffer C. The suspension was centrifuged at 40000xg for 30min. The supernatant was kept (Fraction III, 8.7ml). Step IV: ammonium sulphate (0.28gm/ml) was added to Fraction III. The mixture was kept on ice for 2h, agitated occasionally, and centrifuged at 13000xg for 30min. The pellet was dissolved in 1ml of Buffer C, and dialysed against 200 volumes of the same buffer (Fraction IV, 1.3ml). Step V: Fraction IV (0.6ml) was applied to a Sephadex G-200 column (2x25cm) which had been equilibrated with 400ml of Buffer C. The column was eluted with Buffer C and 0.53ml-fractions were collected. The peak fractions of PhoU protein (Nos. 31-40, Fig. 7.5) were pooled and concentrated by ammonium sulphate precipitation, as above. The pellet was dissolved in 0.2ml of Buffer C, and dialysed against 200 volumes of this buffer (Fraction V, 0.26ml).

Fig. 7.5. Purification of the PhoU protein. A. Profile of elution from Sephadex G-200 as described in Section 7.4. Fractions (0.53ml) were assayed for protein by the method of Bradford (1976). The elution volumes for the standards blue dextran 2000 (BD), bovine serum albumin (BSA) and ^{3}H-leucine (^{3}H-LEU) were determined independently and are indicated with arrows. B. Samples (5µl) of individual fractions (as indicated) were mixed with two volumes of "cracking buffer" (Chapter 2, Section 2.3D), and the samples made up to 20µl with Buffer C (Section 7.4). The samples were heated at 100°C for 2min, and electrophoresed on a 12% (w/v) polyacrylamide gel containing 0.1% (w/v) SDS as described in Chapter 2 (Section 2.5D). The molecular weight standards were phosphorylase b [93 kilodaltons (kDa)], bovine serum albumin (67kDa), ovalbumin (43kDa), carbonic anhydrase (30kDa), and soybean trypsin inhibitor (20kDa).


A
150
100
50
0
PROTEIN, µg/ml
BD
BSA
3H-LEU
20
40
60
80
FRACTION No
B
29 31 33 35 37 39 41 43 45 47 49 51 53 55 57 59 61
kDa
93
67
43
30
20
Dye
⊖
⊕

After chromatography on Sephadex G-200 (Fig. 7.5) the purity of the protein was greater than 99%, as judged by one-dimensional polyacrylamide gel electrophoresis in the presence of SDS (Fig. 7.6). This protein chromatographed on the Sephadex column as a series of polydisperse aggregates, with the peak fraction eluting just after the void volume (Fig. 7.5).

7.5 AMINO ACID ANALYSIS OF PURIFIED PhoU PROTEIN

The purified protein was subjected to automated amino acid sequencing (Chapter 2, Section 2.11A). The first twenty-two N-terminal amino acid residues of the purified protein were:

```
 1                                   10
Met-Asp-Ser-Leu-Asn-Leu-Asn-Lys-His-Ile-Ser-Gly-Gln-Phe-Asn-Ala-

             20
Glu-Leu-Glu-Ser-Ile-Arg
```

This is in complete agreement with the corresponding amino acid sequence deduced from the nucleotide sequence of the *phoU* gene (Chapter 3, Fig. 3.7D). The amino acid composition of the purified protein was determined, as described in Chapter 2 (Section 2.11B), and is presented in Table 7.2 together with the composition deduced from the nucleotide sequence. There is close agreement between the two sets of values.

Fig. 7.6. SDS-polyacrylamide gel electrophoresis of the PhoU protein. Samples (4μg of each) were prepared for electrophoresis by heating at 100°C for 2min in "cracking buffer" (Chapter 2, Section 2.3D), and electrophoresed on a 12% (w/v) polyacrylamide gel containing 0.1% (w/v) SDS as described in Chapter 2 (Section 2.5D). Molecular weight standards (A and G) were phosphorylase b [93 kilodaltons (kDa)], bovine serum albumin (67kDa), ovalbumin (43kDa), carbonic anhydrase (30kDa), and soybean trypsin inhibitor (20kDa). The samples which were purified from cells of strain HR303 (pAN275/cI857) and subjected to electrophoresis included (see Section 7.4): Step I (B); Step II (C); Step III (D); Step IV (E); Step V (F).

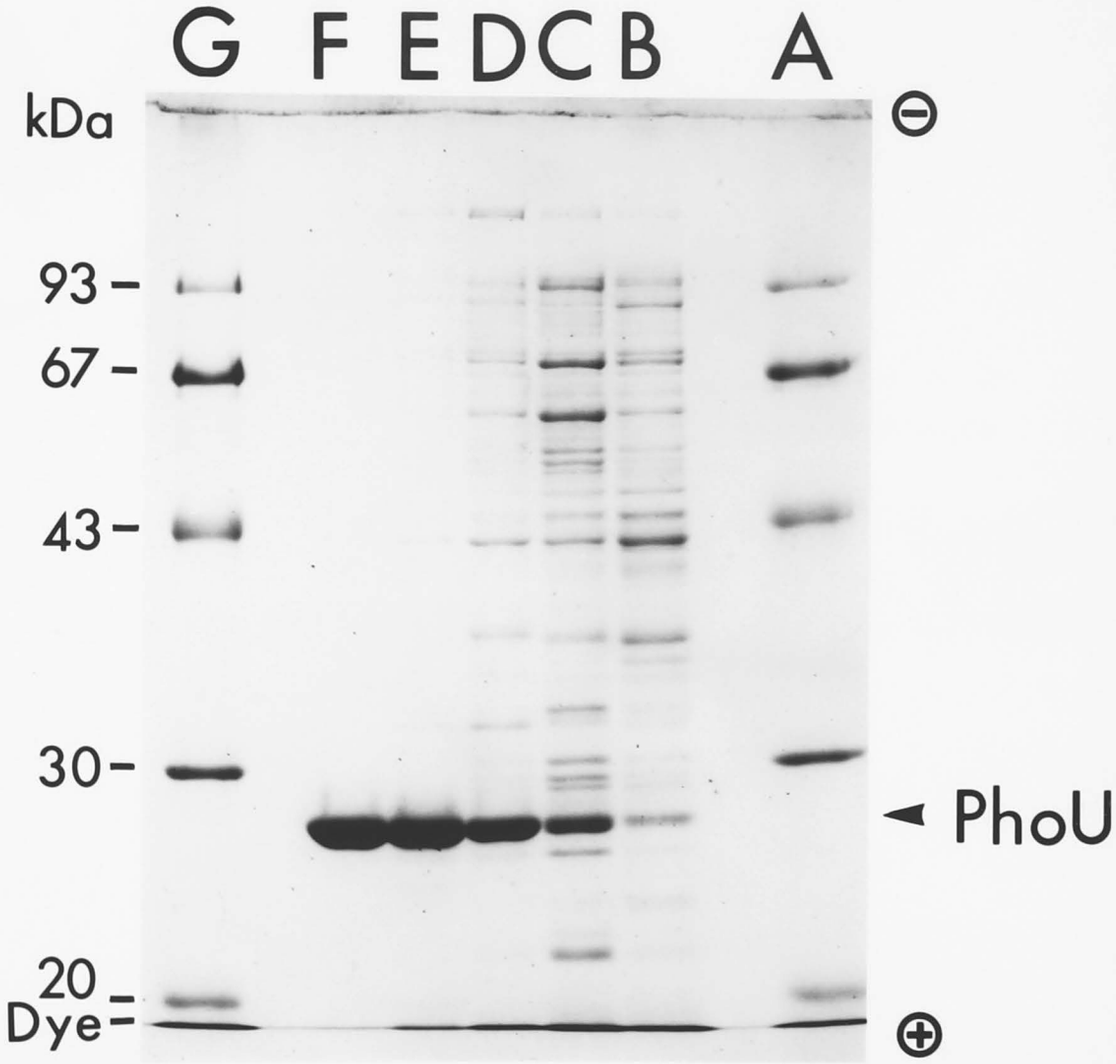

G F E D C B A
kDa
⊖
93
67
43
30
20
Dye
PhoU
⊕

Table 7.2. Amino acid composition of the PhoU protein.

The amino acid composition of the purified PhoU protein was determined as described in Chapter 2 (Section 2.11B).

Amino acid	Number of residues	
	Deduced from DNA sequence	Amino acid analysis of purified PhoU protein
Asn	7	28.6
Asp	22	
Thr	10	9.7
Ser	14	12.9
Gln	14	33.6
Glu	18	
Pro	3	3.5
Gly	12	13.3
Ala	17	17.5
Cys	5	3.9
Val	17	15.9
Met	13	12.2
Ile	20	18.1
Leu	21	21.3
Tyr	5	4.9
Phe	7	6.8
His	6	6.4
Lys	13	13.0
Arg	17	18.5
Trp	0	ND[a]

[a] ND, Not determined.

7.6 DISCUSSION

A procedure has been devised for the purification of the PhoU protein, following its over-production by placing the *phoU* gene under the control of the bacteriophage λ promoter p_L. Determination of the overall amino acid composition and the N-terminal amino acid sequence of the purified protein confirmed the reading frame established for the *phoU* gene (see Chapter 3). The *pstB* gene was also cloned, together with the *phoU* gene, into the plasmid carrying the promoter p_L. The *pstB* gene product was not over-produced to the same extent as the PhoU protein (see Fig. 7.3).

The low recovery (5%) of pure PhoU protein from the lysate of strain HR303 is probably due to the presence in the lysate of a substantial amount of this protein in a non-aggregated form (see below), which was not sedimented by high-speed centrifugation. Further reductions in the overall yield of pure PhoU protein were incurred during the Triton X-100 extraction (see Table 7.1), and in the Sephadex G-200 chromatography where the protein did not emerge as a single narrow peak (Fig. 7.5). Triton treatment prevents sedimentation of other contaminating proteins with the aggregate of PhoU protein, and therefore results in a considerable purification. Attempts to obtain the PhoU protein in monomeric form in high yield without the use of denaturing agents have been unsuccessful (data not shown). Since no assay exists for measuring the activity of the PhoU protein *in vitro*, it is not possible at this stage to determine whether the monomeric or polymeric forms of this protein are catalytically

active.

The procedure for the purification of the PhoU protein described in Section 7.4 is based on the accumulation of the over-produced protein in an aggregated form in the cytoplasm. Preliminary results (not shown) indicate that the presence of such aggregates in "induced" cells of strain HR303 (pAN275/cI857) is determined by the time taken to raise the temperature from 30°C to 40°C. If this time is increased from 10 to 20min, these aggregates are not seen, even though the cells appear to contain the same amount of PhoU protein, and this protein cannot be purified by the procedure described in Section 7.4.

The aggregation of over-produced proteins has been reported by other workers. Thus the over-production of two membrane-bound enzyme complexes of E. coli, the enzyme fumarate reductase (Weiner et al., 1984) and the F_1F_0-ATPase (von Meyenburg et al., 1984), resulted in each case in the accumulation of the over-produced proteins in structures resembling tubules and vesicles. Such structures were presumably derived by invagination of the cytoplasmic membrane.

CHAPTER 8

GENERAL DISCUSSION

8.1 THE PST SYSTEM AND PHOSPHATE TRANSPORT

The work described in this thesis has shown that the pst region of the E. coli chromosome consists of five genes: phoS, pstC, pstA, pstB and phoU. The first four genes are essential for high-affinity Pi transport and for the regulation of the genes comprising the pho regulon (Chapters 4 and 5), while the phoU gene differs in that Pi is still transported in the absence of a functional phoU gene product (Chapter 6). The possible role of the PhoU protein in the Pst system is discussed below. It is proposed that the phoS gene be renamed pstS, so that all components of the Pst system which are essential for high affinity phosphate-specific transport have the designation pst.

The number and distribution of the components which are essential for substrate transport across the inner membrane in the Pst system agrees with those reported for the histidine permease of S. typhimurium (Higgins et al., 1982b) and the maltose transport system of E. coli (see Hengge and Boos, 1983). All three systems consist of a single periplasmic substrate-binding protein and three inner membrane proteins, two of which are hydrophobic and one hydrophilic. The proposed adenine nucleotide-binding fold is located within each of the hydrophilic proteins of these systems.

A model has been proposed (Hengge and Boos, 1983) for the mechanism of substrate transport by the maltose transport system. In this model the inner membrane components (the MalF, -G and -K proteins) are maintained in a conformational state of high energy through the hydrolysis of ATP by the MalK protein. Maltose-binding protein (MBP) carrying bound substrate, but neither MBP alone nor "free" substrate, interacts with the "energized" MalFGK complex of the inner membrane. This complex then undergoes several changes of conformation, each successive change resulting in a conformation of lower energy, and the substrate is ultimately released into the cytoplasm. The hydrophobic inner membrane proteins, MalF and MalG, provide a binding site for the transport of substrate. Hydrolysis of ATP by the MalK protein then occurs, to re-establish the "energized" state of the MalFGK complex, allowing another passage of solute to occur as described above.

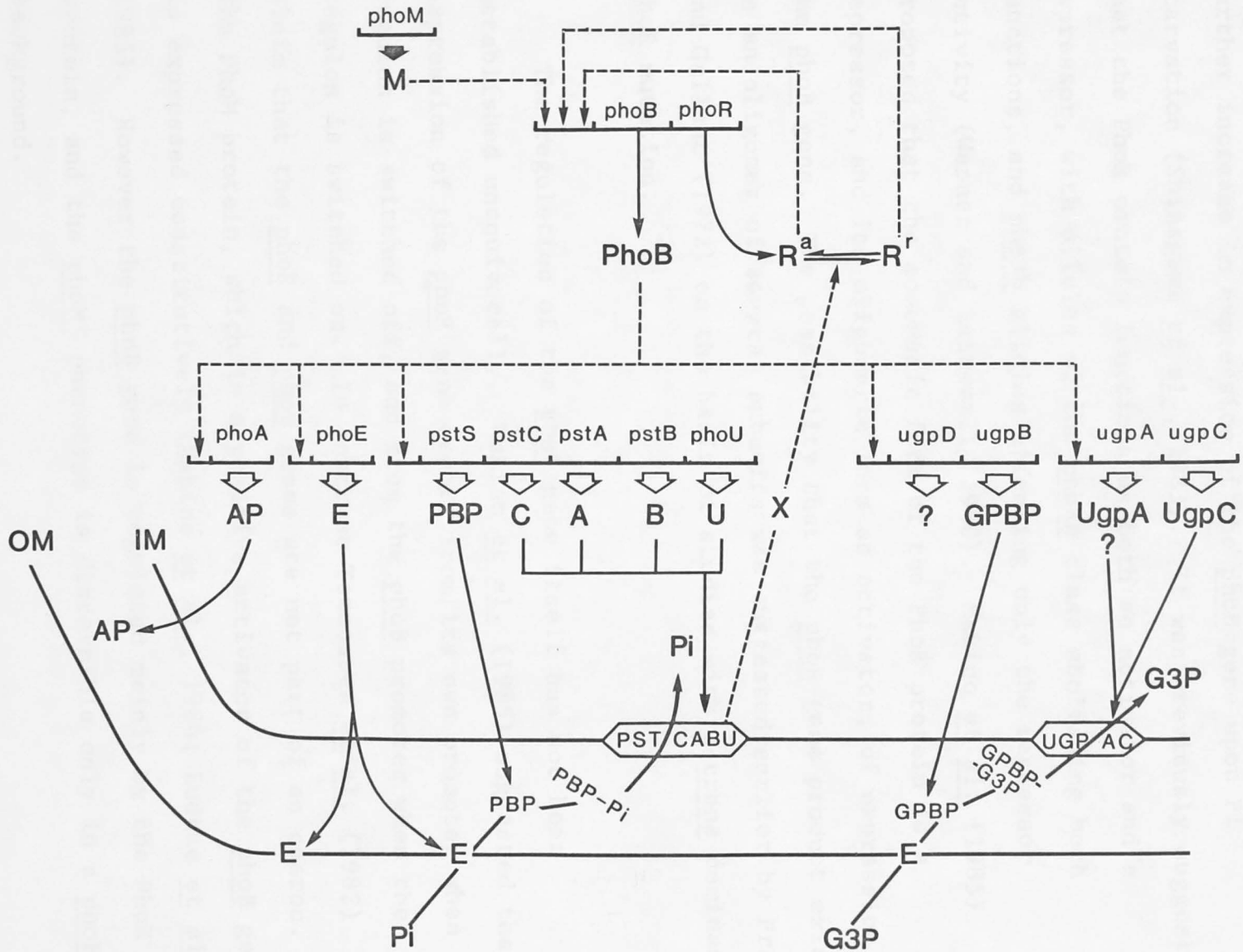
Although the Pst system has not been characterized to the same extent as the maltose transport system, the similarities between the two systems suggest that such a hypothetical description may apply to Pi transport in the Pst system. The PstB protein has the conserved adenine nucleotide-binding fold, and could therefore maintain the energized state of the inner membrane proteins through binding and hydrolysis of ATP. The PstC and the PstA proteins are hydrophobic inner membrane proteins, and could conceivably facilitate the translocation of Pi across the inner membrane.

8.2 ROLE OF THE PST SYSTEM IN THE *pho* REGULON

The Pst system differs from other shock-sensitive transport systems in that it performs the additional function of regulation of gene expression. Since the best-characterized shock-sensitive transport systems contain three inner membrane proteins, while the Pst system has four, the regulatory role of the Pst system may well be related to the function of the fourth inner membrane protein, the PhoU protein. While an interaction between this protein and the other components of the Pst system has not been demonstrated, the *phoU* gene is known to be coordinately regulated, and most likely coordinately transcribed, with the other *pst* genes. Furthermore, the PhoU protein may be required for maximal rates of Pi uptake by the Pst system (Chapter 6).

A model for the regulation of gene expression in the *pho* regulon is presented in Fig. 8.1. Gene expression in the *pho* regulon is controlled by the *phoB* gene product, which is a positive activator for the expression of the *phoA* (Brickman and Beckwith, 1975), *phoE* (Tommassen and Lugtenberg, 1980), *ugp* (Schweizer and Boos, 1985), and *pst* genes (H. Rosenberg, personal communication). In the class of *phoB* mutation designated *phoBa*, which is phenotypically similar to a deletion of the *phoB* gene (Willsky and Malamy, 1976), the genes under the control of the PhoB protein are not expressed during Pi limitation or in a *pho* constitutive background. The *phoB* gene in turn is regulated by the products of the *phoR* and *phoM* genes (Shinagawa *et al.*, 1983; Guan *et al.*, 1983). Two classes of *phoR* mutations, designated

Fig. 8.1. The pho regulon of E. coli. The genes known to comprise the pho regulon are indicated above the horizontal lines with vertical divisions. The corresponding gene products are indicated below each gene by large arrowheads and their general physical location in the outer membrane (OM), inner membrane (IM), periplasmic space and the cytoplasm, are shown by arrows. Broken-line arrows indicate proteins which regulate gene expression or protein configuration. The putative signal "X", provided by the Pst system, affects the equilibrium between the activator (R^a) and the repressor (R^r) forms of the phoR gene product. The products of the ugpA and ugpD genes have not been identified (Schweizer and Boos, 1984). The UgpA protein is tentatively assigned to the inner membrane. A model for the operation of the regulon is presented in Section 8.2. Abbreviations of proteins: A, PstA protein; AP, alkaline phosphatase; B, PstB protein; C, PstC protein; E, PhoE protein; GPBP, sn-glycerol 3-phosphate-binding protein; M, PhoM protein; PBP, phosphate-binding protein; R, PhoR protein; U, PhoU protein. The figure was reproduced with the kind permission of H. Rosenberg.


phoM
M
phoB
phoR
PhoB
R^a
R^r
phoA
phoE
pstS
pstC
pstA
pstB
phoU
ugpD
ugpB
ugpA
ugpC
AP
E
PBP
C
A
B
U
X
?
GPBP
UgpA
UgpC
?
OM
IM
AP
Pi
G3P
PST CABU
UGP AC
PBP-Pi
PBP
GPBP-G3P
GPBP
E
E
E
Pi
G3P

phoRa and phoRb, lead to constitutive expression of the phoB gene, with strains carrying alleles of the phoRb class showing a further increase in expression of the phoB gene upon Pi starvation (Shinagawa et al., 1983). It was previously suggested that the PhoR protein functions as both an activator and a repressor, with alleles of the phoRa class abolishing both functions, and phoRb alleles affecting only the repressor activity (Wanner and Latterell, 1980). Makino et al. (1985) proposed that the monomeric form of the PhoR protein is a repressor, and its oligomeric form an activator, of expression of the phoB gene. The possibility that the phoR gene product exists as an oligomer of several subunits was suggested earlier by Pratt and Gallant (1972) on the basis of studies with a trans dominant phoR mutation.

The regulation of the phoR gene itself has not been established unequivocally. Makino et al. (1985) suggested that expression of the phoR gene occurs from its own promoter when the regulon is switched off, and from the phoB promoter when the regulon is switched on. In contrast Tommassen et al. (1982) claim that the phoR and phoB genes are not part of an operon. The PhoM protein, which is a positive activator of the phoB gene, is expressed constitutively (Makino et al., 1984; Ludtke et al., 1985). However the phoB gene is regulated mainly by the PhoR protein, and the $phoM^+$ phenotype is discernible only in a $phoR^-$ background.

A model that explains the regulation of the pho regulon by the Pst system must account for the following observations:

i) mutations in any of the genes pstSCAB abolish Pi transport and derepress the pho regulon (as indicated by the constitutive expression of alkaline phosphatase activity);

ii) mutations in the phoU gene derepress the pho regulon but do not abolish Pi transport; and

iii) induction of alkaline phosphatase activity in E. coli occurs at an extracellular Pi concentration of 0.6mM and is maximal at concentrations below 0.15mM (Willsky et al., 1973), while the transport constant for the Pst system is 0.16μM (Rosenberg et al., 1977).

It follows from the above that the phoU gene almost certainly plays a central role in the regulation of the pho regulon by the Pst system. It is also clear that induction of the regulon by Pi limitation or by a mutation that abolishes Pi transport is not directly connected to the decreased flux of Pi through the Pst system.

The presence of a low amount of phosphate-binding protein (PBP) in cells grown in media containing phosphate at concentrations which repress the synthesis of alkaline phosphatase has been reported (Garen and Otsuji, 1964; Willsky and Malamy, 1976). Since the pst genes, including phoU, probably comprise an operon (see Chapter 4), comparable amounts of the other components of the Pst system would also be present under such conditions. It is suggested here that the Pst system regulates gene expression in the pho regulon by providing a signal (designated "X" in Fig. 8.1) under conditions of excess Pi

which can affect the equilibrium between the putative repressor and activator forms of the PhoR protein in the cytoplasm. It is suggested that a low-affinity site capable of interacting with external Pi located within the Pst system cooperates in the production of the signal "X", in addition to the requirement that Pi be flowing through the Pst system. This site would comprise portions of the inner membrane Pst proteins that are exposed to the periplasm. Therefore it is not likely that the low-affinity site consists solely of the PhoU protein, as the hydrophilic nature of this protein suggests that it is located on the cytoplasmic face of the inner membrane. Treptow and Shuman (1985) in their study of maltose transport independent of the MBP reported the presence of a low-affinity ($Km^{app} \approx 2mM$) substrate-binding site comprising the MalF and MalG proteins which are located in the inner membrane.

It can be envisaged that the putative low-affinity site of the Pst system alters its conformation upon binding/release of Pi, and that the conformation of this site and the flow of Pi through the Pst system would control the formation of "X". At extracellular concentrations of Pi in excess of 0.6mM, the binding of Pi at this site would favour production of "X". The PhoR protein would exist mainly in the repressor form, and the phoB gene, and consequently genes under its control, would be expressed at a low level. When the concentration of extracellular Pi is in the region of 0.1-0.6mM the low-affinity site would not bind Pi, and the conformation of this site would not favour formation of "X". Consequently the equilibrium of the PhoR protein would be shifted toward the activator form. The

phoB gene would be "switched" on, resulting in a high level of expression of the genes under the control of the PhoB protein. A mutation in any of the genes encoding the transport function of the Pst system would abolish the flow of Pi through the system. Some pst mutations could also affect the low-affinity site. In either case it follows from the discussion above that the synthesis of the signal "X" would decrease. Consequently the genes under the control of the phoB gene would be constitutively expressed, as in the case of induction by Pi limitation. Mutations in the phoU gene which inactivate the PhoU protein will abolish the provision of the signal "X", as this protein either forms part of the signal or is involved in its production (see below). In either case the ultimate effect would be constitutive expression of the genes comprising the pho regulon (see above).

The nature of the signal "X" may only be speculated upon. By analogy with the regulatory properties of factor III^{glc}, a peripheral inner membrane protein of the phosphoenolpyruvate:sugar phosphotransferase system (see Chapter 1, Section 1.3B), "X" could be the phosphorylated form of the PhoU protein. Its phosphorylation may be catalysed by the transport-related components of the Pst system utilizing energized Pi being transported through this system and could conceivably be affected by the presence of bound-Pi in the low-affinity site. The phosphorylated PhoU protein could bind to the repressor form of the PhoR protein, and prevent formation of the activator form of this protein. When the extracellular concentration of Pi is low, or if there is a mutation in any of the genes encoding the transport-related components of the Pst

system, the PhoU protein will exist mainly in the non-phosphorylated form. Accordingly the activator form of the PhoR protein will accumulate, and switch on the expression of the phoB gene, and genes under the control of phoB. The loss of the low-affinity site within the Pst system by mutation would have a similar effect.

Consistent with the above proposal is the observation that the PhoU protein is weakly attached to the inner membrane (see Chapter 4). It is conceivable that after phosphorylation this protein detaches from the membrane, and can modulate the equilibrium between the monomeric and oligomeric forms of the PhoR protein.

On the other hand, "X" could be derived from an energized form of Pi transported through the Pst system and from an intracellular effector, in a reaction catalysed by the PhoU protein. The phosphorylated effector would act in a manner analogous to that proposed for the phosphorylated PhoU protein (see above). It is clear that "X" can not be Pi per se. Strains carrying pst mutations in a pit$^+$ background are pho constitutive, even though a normal level of Pi is maintained in the cytoplasm of such strains by the operation of the Pit system.

The possibility that the PhoU protein regulated gene expression in the pho regulon, by binding to DNA as an activator or repressor, was investigated. A general model that explains how proteins bind to specific DNA sequences has been proposed on the basis of mutational, biochemical and crystallographic analysis of several DNA-binding proteins (see Pabo and Sauer,

1984). A homologous segment, consisting of two α-helices separated by a sharp bend, has been described for each protein. It has been proposed that one of the helices (designated the "recognition helix") is involved in a specific interaction with the DNA sequence of the operator, while the other helix ("stability helix") stabilizes this interaction with non-specific contacts to the phosphate backbone of the DNA double helix. The secondary and tertiary structure of the PhoU protein was predicted from its deduced amino acid sequence (Chapter 3) by the method of Chou and Fasman (1978). Two segments, each consisting of two α-helices linked by a β-turn, were identified (data not shown). However, it was not possible to identify a DNA-binding domain in this protein, because the segments, and in particular their respective "stability helices", did not contain sequences known to be homologous to several DNA-binding proteins (Kelley and Yanofsky, 1985). In order to establish a repressor or activator role for the PhoU protein in the regulation of gene expression in the pho regulon, the ability of this protein to bind in a specific manner to DNA fragments will have to be demonstrated in vitro.

The model for regulation of gene expression in the pho regulon in Fig. 8.1 does not account for two experimental observations. Firstly, an increase in the constitutive expression of alkaline phosphatase (Garen and Echols, 1962a; Shinagawa et al., 1983) and the Ugp system (Schweizer and Boos, 1985) is seen in strains carrying phoRb or pst mutant alleles following Pi limitation. I have made a similar observation for the Pst system in strains carrying phoRb or phoU mutations (B.

Surin and H. Rosenberg, preliminary results). The increase in each case is less than the increase above the repressed levels of the corresponding activities in a pho constitutive background or during Pi limitation. Shinagawa et al. (1983) reported that the increase in activity of alkaline phosphatase in a pho constitutive background upon Pi limitation coincided with an increase in the expression of the phoB gene. These observations suggest that a mechanism (s) other than that involving the signal "X" (Fig. 8.1) regulates the level of the activator form of the PhoR protein. It is conceivable that a hitherto unknown pathway not involving the PhoR protein can regulate expression of the phoB gene in response to Pi limitation.

Secondly, there is a class of phoB mutation, phoBb, in which alkaline phosphatase is not synthesized (Garen and Echols, 1962b; Willsky and Malamy, 1976) while the Pst system is constitutively expressed (H. Rosenberg, personal communication). Although the DNA sequences upstream of the phoA and the pstS genes show a very high degree of homology, there are sequences upstream of the pstS gene which are not present in the corresponding region of the phoA gene (see Chapter 3). A promoter was tentatively identified in these sequences exclusive to the pstS gene. It is likely that the more complex structure of the pstS promoter region confers on the pst genes regulatory properties which are not shared by the phoA gene. The nature of the expression of the phoE and the ugp genes in the phoBb background has not been investigated. The phoBb class of mutation clearly has to be resolved to obtain a more accurate understanding of the regulation of gene expression in the pho regulon.

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